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PREFACE

The cordial reception given to the first volume of the *Review* was at once a measure of the very real need for a volume devoted to a periodic survey of advances in plant physiology and a tribute to the reviewers for the excellent manner in which they fulfilled this need. The present volume attempts to repeat the general pattern introduced by the first: a survey of progress made in the various branches of plant physiology and associated fields with emphasis on critical evaluation rather than on encyclopedic comprehensiveness in reviewing publications. The necessity of confining the reviews to the assigned space forces the reviewers to exercise selection in covering the literature. This regrettable lack of completeness of coverage in a particular volume is alleviated somewhat by the policy of rotating reviewers, who labor under no rigid calendar restriction in drawing attention or adding stress to older papers whenever a fresh point of view or a change of emphasis demands it. It is hoped that complete coverage of the field of plant physiology will be attained over a period of several years rather than in a single year.

Some concern has been expressed in certain quarters that the launching of this *Review* will tend to dissociate plant physiologists from certain aspects of biochemistry and animal physiology. This is indeed not our expectation. Apart from the purely technical limitations of space which made it increasingly difficult to include plant physiological topics in the *Annual Review of Biochemistry*, it was felt that the creation of this *Review* would permit a full treatment of the contributions and associations between various branches of plant physiology and other sciences, and in this manner help the student and research worker to keep abreast with new approaches and current concepts influenced by allied fields. It is our hope that the present volume, as well as its predecessor, has in some measure attained this objective.

We wish to express our appreciation to all who have made suggestions as to the contents and authors for future volumes and we hope that we may look forward to continued cooperation from our colleagues. Our sincere thanks are due to the reviewers to whose efforts this volume owes its existence. We are further indebted to Dr. H. B. Steinbach and to the Editor of the *Annual Review of Physiology* for permission to reprint the review on Permeability, thus making a discussion of this topic from a new point of view readily accessible to our readers. We also wish to acknowledge the loyal services of our editorial assistants, Joyce V. Fairweather, Robbie Bass, Barbara C. Darneal, Carol F. Kupke, and Ruth H. Swan, as well as the help given us by our printers, the George Banta Publishing Company.

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ERRATA

Volume 1

page 177, line 31: *for* ($W=T=O$) *read* ($W=T=0$)

page 177, line 32: *for* ($E=O$) *read* ($E=0$)

page 182, Ref. 50: *for* (1939) *read* (1945)

page 131, 4th line from bottom: *omit* "naphthaleneacetic acid and"

page 126, lines 28 and 31; page 359, line 4 on left: *for* *Hybiscus* *read*
Hibiscus

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MECHANISM OF ABSORPTION AND TRANSPORT OF INORGANIC NUTRIENTS IN PLANTS

BY R. N. ROBERTSON

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Industrial Research Organization, Homebush, Australia*

The absorption, movement, and function of electrolytes are of general biological importance and are commanding considerable attention at present. Outstanding problems range from the relatively straightforward ionic adjustment resulting from exchanges to the complicated problems, like secretion in animals and accumulation in plants, in which metabolism is involved. This article attempts not only to review the recent work on plants, but also to bring together sufficient evidence to point to lines along which work may develop. Nutritional functions of the various elements are mentioned only insofar as they assist in discussion of absorption or transport. While this is the first review of this title in the *Annual Reviews*, the problems have been dealt with under mineral nutrition in plants in the *Annual Review of Biochemistry* for some years; recent articles were by Lundegårdh (75), Burström (25) and Wadleigh (135). Mulder (93) in the *Annual Review of Plant Physiology* also reviewed recent work. A general review of these problems in biology was written recently by Ussing (131), a review of some aspects of plant accumulation and animal secretion by Robertson (107), and of accumulation in plants by Robertson (106). The article by Stout & Overstreet (125) may be consulted for a discussion of the soil in relation to absorption and that by Biddulph (14) for a discussion of translocation of inorganic nutrients.

Terminology.—The study of salt absorption and transport, like many other fields in biology, has suffered severely from a lack of agreed terminology. In both botany and zoology, the term "membrane" has, for instance, been used to define structures ranging from multicellular layers, e.g., frog skin, to those probably only a few molecules thick. It seems desirable to state some definitions, not only for clarity in attempting to co-ordinate the different hypotheses from the various schools, but also in the hope that it may lead to some standardisation. The definitions given below agree substantially with those of Broyer (22).

"Absorption" and "uptake" are used as general terms covering the entry of a substance into any plant cell, tissue, or organ by any mechanism.

"Accumulation" is used for the entry of a substance against a concentration gradient, and is applied particularly to the concentrating of both ions of the salt. Accumulation implies expenditure of energy by the cell.

The term "secretion," more widely used in animal than in plant physiology, is here used to define the transfer of a substance to a higher concentra-

tion outside the cell, tissue, or organ. Secretion has, however, been used by some plant physiologists for the transfer of ions from the cytoplasm to the vacuole.

"Leakage" means the loss of a substance from a cell, tissue, or organ where it is in high concentration, to the exterior where it is in low concentration.

Movement of substances may occur by "diffusion," if they are free in solution and move with the concentration gradient, or by "active transport," if they are attached, temporarily at least, to some constituent which itself may move. The active transport process is independent of the concentration gradient of the substances being transported and is concerned in both accumulation and secretion.

In all living organisms we find evidence of the independent entry of anions and cations into cells. The presence of indiffusible ions within the cell leads to the establishment of the "Donnan equilibrium" which may be defined as the unequal distribution of ions between two phases, resulting from the presence of an indiffusible ion in one phase. Adjustments between the diffusible ions already present in a cell or tissue and other ions applied to the external surface are known as processes of "ion exchange," and permit the entry of large quantities of one ion of a salt, sometimes referred to as "non-metabolic accumulation" leading to a high concentration of one ion.

Ions held by polar linkages to charged groups on cellular structures are said to be held by "adsorption" and are available for exchange. Some ions enter into the formation of organic molecules. Such ions are said to have undergone "combination," a general term for ions removed from solution as ionic species. Metal ions, particularly of higher atomic weight, may enter into organic molecules by the formation of co-ordination or chelate compounds, for which the term "metal-organic complexes" will be used.

Great confusion has existed in the terminology of the plant cell, particularly with regard to the use of the word "membrane." The protoplasm lining the cell wall is referred to as the "cytoplasm." The outer surface (the plasmalemma) is referred to as the "surface of the cytoplasm" and the inner surface adjoining the vacuole is referred to as the "tonoplast." These terms are used advisedly, since there is some doubt as to the existence of the plasmalemma as a distinct structure, though the presence of the tonoplast seems better established.

The term "membrane" is confined to structures probably a few molecules thick with special properties due to molecular orientation.

DIFFUSION

Simple diffusion of both ions of a salt does not seem a very important process in absorption by cells. There is evidence suggesting that diffusion is slow because of the high resistance of the cytoplasm to movement of both ions of a salt, due either to the presence of lipid membranes discussed in detail by Davson & Danielli (45) and Danielli (43), or to diffusion being

retarded by the presence of indiffusible ions of either charge. Consideration of the Donnan equilibrium shows that the presence of indiffusible anions such as the proteins in cells will exclude the entry of anions, though at the same time there may be free exchange of cations. The net effect is that the resistance to diffusion of salt in such a system will be high [Briggs & Robertson (20)]. The organisation of lipid membranes may be important too, but so far, the only well established membrane structure in the cytoplasm is the tonoplast on the surface of the vacuole; it is questionable whether resistance to movement into the cytoplasm from outside the cell is due to a lipid membrane.

Though some simple diffusion takes place in wet cell walls and water-filled intercellular spaces in tissue, it is complicated by the nonmobile anions of the walls and cytoplasm. This has been demonstrated in a disc of carrot tissue which behaves as a structure containing nonmobile anions [Briggs & Robertson (20)]. In such structures, the apparent diffusion coefficient falls with decrease in the ratio of the concentrations of the mobile ions to the nonmobile anions. Similar considerations can be used to relate observed potential differences to concentration differences within and without membranes or cytoplasm, but unfortunately concentrations within parts of the living cell are largely unknown. Eriksson (50) discussed diffusion of univalent ions through a negatively charged homogeneous membrane, through a positively charged membrane, through a negatively charged membrane under the influence of oxidation-reduction processes, and of univalent anions through a negatively charged membrane in the presence of mono- and divalent cations and anions. As these theoretical treatments become more widely used, it will be possible to extend the type of work reported by Mattson, Eriksson, Vahtras & Williams (87) and discussed in the next section.

ION EXCHANGE AND DONNAN EFFECTS

Ion exchange can occur wherever a mobile ion is held by a polar linkage to another ion in the cell, and depends upon (a) the concentration of the adsorbing ion, (b) the concentration of the adsorbed ion, (c) the concentration of competing mobile ions of similar charge to the adsorbed ion, and (d) the relative affinities of the adsorbing and of the competing ions. Ionic exchange complicates the relative uptakes of nutrient ions by plants grown in varying conditions. The occurrence of the inorganic nutrients in the soil, and its relation to roots, has been discussed recently by Stout & Overstreet (125).

Factors influencing availability of plant nutrients in synthetic ion exchange materials have been investigated by Arnon & Meagher (8). Mattson (88) showed that kaolin and bentonite affect differently the proportions of potassium and calcium ions absorbed from a quartz sand mixture. Differences in the uptake of mono- and divalent ions were attributed to properties of the plant colloids. Mattson, Eriksson, Vahtras & Williams (87) showed that neutral salts increased uptake of the phosphate ion by pea, rye, and bar-

ley seedlings. This effect was explained by the Donnan equilibrium. They suggested that the presence of salts allowed a greater concentration of the anions of the external solution in the region of the anions ("acidoid") of the root surface. While these considerations would apply to the cytoplasm itself where the prevailing constituents are anionic, Mattson *et al.* suggested that the strongly acidic groups of the pectins in the cell wall were more important. In either case, the results are due to the Donnan equilibrium adjustments such as occur at all cell surfaces. Donnan equilibria have also been studied by Elgabaly & Wiklander (48, 49). They examined the effect of exchange capacity of the clay minerals kaolin and bentonite on the uptake of sodium and calcium by excised barley roots. The differences between species in their capacity for absorption could be explained on the Donnan theory when adequate allowance was made for the differences in "acidoid" content in the plants.

Exchange is important when the accumulation from a solution of a single salt is being followed, particularly in the early stages, and accounts for the unequal uptake of anions and cations [Lundegårdh (79), Rees (102)].

In the study of the intact plant, further complications arise because of the varying exchange capacities of cells in different regions. Further, the phenomenon of exchange is not static, but the exchange ratios are likely to be altered by the effects of the ions' own activity within living cells [Ulrich (130), Shear, Crane & Myers (116), Wallace, Toth & Bear (138)]. In intact guayule plants [Coil & Slattery (38) and Coil (36, 37)] large differences in potassium content, obtained by varying the supply, were compensated by reciprocal differences in other cations. Relatively little sodium was absorbed. A large proportion of the absorbed potassium, calcium, magnesium, nitrogen, and sulphur was, as might be expected, in the leaves.

Bower & Wadleigh (19) showed that different species varied widely in response to sodium on the ion exchange resins in the culture medium. While the calcium and magnesium per unit weight of root was constant, there was a marked decrease in potassium accumulation in roots with increasing exchangeable sodium percentage. This they interpreted as being due to potassium of colloids in contact with roots becoming less exchangeable as the adsorbed complementary ions moved from calcium to sodium. While sodium stays in the roots, potassium moves readily to the tops, and a potassium deficiency shows in the roots with a calcium deficiency in the tops. An increasing proportion of exchangeable sodium in the culture medium increased the sodium content in the leaves and led to calcium deficiency in the absorbing cells of the root; roots so injured may lose their selective capacity to retain sodium which thus passes to the leaves. With bean plants grown in a nutrient solution containing a high concentration of sodium ions, however, much sodium occurred in the roots, some in the stems, but very little in the leaves [Gauch & Wadleigh (53) and Wadleigh & Bower (136)].

Smith, Reuther & Specht (119), with different root stocks in Valencia orange trees, found highly significant differences in the percentages of nitro-

gen, potassium, calcium, magnesium, manganese, copper, zinc, and iron in the leaves, though the sodium content was not affected. Larger variations were induced in the concentrations of micro- than of macro-nutrients.

COMBINATION

The entry of ions by chemical combination is, of course, similar to their entry by adsorption. We must expect a range from the loosely held ions of salt formation to the tightly held ions characteristic of the formation of metal-organic complexes, such as iron in the iron-porphyrins and magnesium in the chlorophylls. Clearly, wherever an ion enters into the formation of a compound so that its activity in solution is reduced, the gradient will be favourable to the entry of further ions of the same kind. At present, our knowledge is limited but it is probable that in addition to magnesium and iron, copper, zinc, manganese, molybdenum, cobalt, and aluminum enter into the formation of such compounds. Hutner (62) has discussed the probable importance of chelating compounds at the surfaces of unicells.

It is possible that in accumulation the initial uptake of ions is more than an exchange of ions held by electrovalent linkages. Combination probably occurs with transient metabolites not yet identified. The marked difference between the uptake by live roots and killed roots illustrates that simple exchange on stable compounds is only part of the story [Overstreet & Jacobson (100) and Jacobson & Overstreet (63)], though it is not yet clear how much of this difference is due to combination and how much is due to the rapid accumulation of ions in the vacuoles of the living cells.

Phosphate.—Phosphate is apparently largely bound as organic ester phosphate either on the carbohydrate or in the phosphate transport system [Wildman, Campbell & Bonner (142)]. Rabideau, Whaley & Heimsch (101) studied the uptake of radioactive phosphorus (P^{32}) in maize plants. The hybrid with the largest root system absorbed most phosphorus and in all plants the highest concentrations of phosphate were in regions of great metabolic activity.

Sacks (114), reviewing the available evidence, indicates that in heart, muscle, and liver, phosphate enters the cell by formation of an organic phosphate compound in the cell membrane. It seems that uptake by the plant cell or root is also dependent upon combination of the ion with an appropriate organic carrier. Alberda (1) made a detailed study of the uptake of phosphate by maize plants under different salt conditions, and suggested that the protoplasm of the root has a definite combining power for phosphate. In short experiments, the absorption of phosphate with both high salt and low salt plants is independent of the other anions in the culture solution, and this binding is specific in the sense that it can be replaced only by an isotope or chemically similar ion. Humphries (60, 61) has studied the uptake of ions by roots excised from plants deficient in a particular ion. He found preferential absorption of the elements in which they were deficient. Thus, high salt roots previously deficient in phosphorus took it up readily.

Aronoff (9), using radiophosphate on soybean roots, has shown that the primary product of phosphate assimilation by roots is fructose-1,6-diphosphate together with some phosphorylated organic acids. MacEwan (83) has investigated the uptake and movement of radiophosphorus in squash plants. He found rapid uptake from the nutrient solution and a lag in the entry of phosphate into the leaves. Radiophosphorus uptake by the leaves seemed to depend on the presence of photosynthetic substances. Street & Lowe (127) and Lowe (71) have shown that the mechanism of sucrose absorption by excised tomato roots is associated with phosphorus uptake and that phosphorolysis occurs at the root surface.

Kramer & Wilbur (67) showed that the mycorrhizal roots of pine (*Pinus taeda* and *Pinus resinosa*) took up P^{32} from the external solution and that the mycorrhizal portion absorbed more than the nonmycorrhizal portion. Melin & Nilsson (89) exposed only the mycorrhizal hyphae to the P^{32} and showed that the hyphae transported the phosphorus.

Nitrogen.—In this review it is not possible to discuss the full bearing of current hypotheses of nitrogen metabolism on the uptake of nitrogen, either in the form of the ammonium or nitrate ions. This was discussed by Burström (24); nitrate entering the plant is reduced to nitrite and ammonia before synthesis of organic compounds. Some free nitrite and ammonium may occur, but nitrate if well supplied accumulates and is reduced slowly. Nitrate accumulation probably follows the same mechanism as that of other ions, but it is certain that its metabolic combination results in alterations of concentration, favouring its further entry. Oxygen inhibits combination but not accumulation of nitrate by wheat roots [Nance (96)]. Willis (143) showed that in seedlings grown under high light intensities in solutions lacking nitrogen, carbon dioxide production is increased very rapidly by addition of ammonium, nitrite or nitrate in concentrations from 0.004 *M* to 0.002 *M*. The respiratory quotient changes distinctively depending on the form of nitrogen supplied, and analytical data for sugars, organic acids, and nitrogen fractions indicates increased glycolysis associated with the synthesis of amino acids and amides, particularly glutamine. Burström (27, 28, 29), showed that *n*-diamylacetic acid applied to roots checked the elongation of the piliferous layer and caused it to be shed. Nitrate combination was decreased and finally checked completely. This inhibition was counteracted by manganese and iron but not by molybdenum, suggesting that in the normal root, manganese may counteract some naturally occurring substance behaving like *n*-diamylacetic acid.

Burström's view that the uptake of nitrogen and the formation of amino compounds in roots is associated with manganese, is supported by the work of Jones, Shepardson & Peters (65). Humphries (60) showed that the rate of uptake of nitrogen in excised roots increased with the degree of deficiency; effects on the carbohydrate and respiratory metabolisms were observed. The metabolic activity of an organ thus clearly affects its uptake of any form of nitrogen.

Manganese and iron.—Sideris & Young (117) showed that not only was the relative concentration of iron and manganese important, but also the form in which the nitrogen was supplied. Iron concentrations were greater in tissues in ammonium than in nitrate cultures, while manganese concentrations were greater in cultures supplied with nitrate. This was thought to be due to the antagonism between the manganese and ammonium ions. These results are an interesting example of the chemical properties of two ions influencing their uptake and movement in the plant, because the chlorosis which occurs at high manganese concentration is explained on the hypothesis that manganese substitutes for iron in protoporphyrin 9, the chlorophyll precursor. Sideris (118), using radioactive iron (Fe^{59}), has shown that most iron removed from the nutrient solutions was deposited in the roots, particularly in cultures supplied with manganese. Transfer of iron from roots to leaves was considerably lower in cultures with manganese. It was suggested that most of the iron remained in combination with the protein fractions of the cells. Millikan (91), using flax, has shown also that the effects of manganese or molybdenum are dependent upon the form in which the nitrogen is supplied.

Biddulph (13, 14) has demonstrated that iron ($\text{Fe}^{55,59}$) and phosphorus (P^{32}) affect the absorption of each other, and that the pH of the nutrient solution is also important. When phosphorus was not present in the medium, low iron roots absorbed much iron, thus explaining how withholding phosphorus from the medium can cure iron chlorosis. The absorption of phosphorus was greatest from solutions low in iron. There was evidence for the immobilisation of both ions at the root surface and analysis of the precipitate showed that below pH 6 it was predominantly ferric phosphate. Both ions may also become immobilised in the veins.

Other micronutrients.—Our knowledge of the uptake of such nutrient constituents as boron, aluminum, copper, zinc, manganese, and cobalt is small. We have little knowledge of the occurrence of characteristic metal-organic complexes in the plant. Some of the interactions of these elements discussed by Wallace & Hewitt (137) and by Millikan (90) raise the question of the way in which excess of one such element may result in the replacement of another from what is apparently an essential constituent.

Work on the chemistry of metal-organic complexes is still relatively new. Only recently have satisfactory methods been developed to study the stability constants of the compounds which chelate with metals [Calvin & Bailes (32), Albert & Gledhill (2), Maley & Mellor, (86)]. Chelation by amino acids has been little studied and practically nothing is known about other chelating compounds in living cells. Levitt (69) has reported the occurrence of iron, copper, zinc, and manganese in the protein of potato. Chenery (34) suggests that some plants do not change colour in the presence of aluminum because the cell sap is too low in acid to allow translocation of this element. Wallihan (139) investigated the effect of aluminum on phosphate movement and metabolism in clover plants and concluded that there was no evidence for earlier

suggestions of aluminum interference with phosphate movement and metabolism. Nickerson & Zerahn (98) showed that radioactive cobalt is accumulated by dividing yeast cells, and that the cobalt so accumulated is not free to diffuse from the cells.

Wood & Sibly (144) examined the absorption and movement of zinc in oat plants. Large increases in the amount of zinc in the soil did not result in proportional increases in the plant nor, above a threshold level, in increases in the yield. The leaves contained 20 to 30 per cent of the total zinc, largely in the chloroplasts, which was not translocated during senescence, and could not be removed by dialysis of the dead material. The amount in the roots increased up to the time of grain development, after which it was translocated to other organs.

Sodium and potassium.—A great difficulty has been to explain how living cells distinguish between such similar ions as sodium and potassium. It has perhaps been too readily assumed that the similarity between these ions involves similar affinities for the substances with which they combine either temporarily or permanently in the cell. Reference will be made in a later section to the work on *Escherichia coli* (40, 104), in which it appears that phosphate compounds are formed which have a low affinity for sodium and a high affinity for potassium. Scott (115) suggested that in *Chlorella*, phosphate may be important in the uptake of potassium. Mullins (94) showed that the addition of phosphate to sea water increased the uptake of potassium by *Valonia*.

The possibility of differences in the combining power of proteins for even such similar ions must also be considered. Stratmann & Wright (126) have recently shown that equine haemoglobin had a markedly greater affinity for potassium than for sodium. Olsen (99) showed that about 30 per cent of the potassium was retained in beech leaves that had been frozen and afterwards thawed out; it was assumed that this potassium was bound to the proteins.

Uranium.—Uranium in low concentration inhibits the glucose metabolism of the yeast cell. Barron, Muntz & Gasvoda (11) suggested that uranium might work at the surface of the cell because they found differences between the effect of the uranium on yeast cells and on yeast juice or enzyme systems. Rothstein & Larrabee (113) suggested that the uranium acts by forming complexes with anion groups at the cell surface. Rothstein, Frenkel & Larrabee (112) suggested that the percentage and the dissociation constant of these active groups could be calculated.

In the whole plant.—The complexity of the problem of absorption by the whole plant is further illustrated by the work of Alberda (1) and Van Anel, Arisz & Helder (132). Following earlier observations, that in darkness exosmosis of phosphate and potassium occurred, Alberda was able to relate the uptake rate of phosphate to the sugar content of the roots. Van Anel *et al.* (132) showed that the uptake of potassium was dependent on exposure of the shoot to light but was not increased by the addition of sugar to the nutrient solution. They did not find exosmosis of phosphate in darkness. There

was distinct exosmosis of potassium, especially when the dark period lasted for some days. This might have been associated with the transport of potassium from the shoot when growth was checked in darkness.

In discussing the absorption of individual ions, it must be recognised that exchange, combination, and accumulation will all be high in places or periods of high metabolic activity. This has been stressed by Steward and his collaborators. In his survey of application of radioactive isotopes, Steward (122) discussed the centres in the plant where processes of growth and metabolism are connected with salt accumulation. Recently his work has been concerned with the growth of carrot tissue in culture media suitable for accumulation studies [Caplin & Steward (33) and Steward & Millar (122a)]. Most workers on salt accumulation agree with Steward's view that the uptake of ions is intensified in actively growing regions.

Cooper (39) has recently published an article which reviews his earlier suggestions on the effects of energy properties of some plant nutrients on availability, on rate of absorption, and on intensity of certain oxidation-reduction reactions. This work, which has met with the trenchant criticism it deserves [Leeper (68)], does not help in understanding the mechanisms of uptake by plants; the criticism by Burström (25) still stands.

ACCUMULATION

The driving force.—Since the recognition that accumulation of ions in solution against a concentration gradient requires the expenditure of energy by the cell, there have been various theoretical and experimental attempts to connect the accumulation process with the energy expenditure in respiration and to suggest the nature of the carrier involved. The principles involved in the expenditure of chemical energy performing osmotic work have been discussed in a valuable theoretical paper by Franck & Mayer (51). The principles of secretion have been dealt with by Davies & Ogston (44). Where secretion or accumulation implies movement of electrolyte to a higher chemical potential, constraint must be placed on at least one ion. Since a single ion cannot be secreted alone because of the requirement of maintenance of electrostatic neutrality, a second ion follows the one upon which constraint has been placed. Davies & Ogston distinguish the secretion of the ion undergoing constraint as "primary" secretion and that of the ion which follows to maintain electrostatic neutrality as "secondary" secretion. Initially, however, the metabolism of the cell must be organised in such a way as to produce and separate electric charges.

Beginning with the observations of Lundegårdh & Burström (82), the theory of the dependence of accumulation on anion respiration has been developed. Other workers, while recognising the same effect on respiration, have called it salt respiration, to avoid attributing the effect specifically to the anion. The two terms have become interchangeable, though Lundegårdh has always maintained that the relation between this respiration and accumulation is specifically due to the transport of anions from the external solu-

tion by the respiratory system, and that there is no direct effect of the respiration on the movement of cations, which move only because of the hydrogen ions liberated in the cell [cf. Wadleigh (135)].¹ There is still no evidence against Lundegårdh's views. Not all tissues show an increase in respiration on addition of salt; thus roots from a culture solution or storage tissues just cut require some time in aerated distilled water before an increase is observed on addition of salt. Such tissues also increase in capacity for absorption [Rees (102)].

Discussing the quantitative relations between respiration and salt absorption, Lundegårdh (78) recognises two types of respiration other than anion respiration. The cyanide-stable ground respiration, which has been recognised for some time, is not thought to participate in salt accumulation but to be associated with a system not containing cytochrome and possibly with oxidations involving manganese. The "third respiration" recognised by Lundegårdh is present only in the lower 30 mm. of the root and is inhibited by 0.001 *M* hydrocyanic acid, but not by 0.001 *M* alkali potassium cyanide.

Lundegårdh (73) proposed that the anion respiration was the driving force for accumulation because, at the cytochrome stage, hydrogen ions and electrons derived from the hydrogen atoms of respiratory substrates were separated. The electrons passed out by the cytochrome system; the hydrogen ions, as far as was known, were liberated into the intracellular environment. Lundegårdh suggested that the cytochrome, which carried the electrons in one direction because of the change in valency of the iron atom, could carry anions in the opposite direction. Simultaneously, the hydrogen ions liberated as the result of dehydrogenase activity would pass to the exterior of the cell and cations from the exterior would enter the cell by exchange with those hydrogen ions.

Such a mechanism would set an upper limit to the rate at which accumulation could take place for a given salt respiration rate. Robertson & Wilkins (108, 109) showed that in carrot tissue the accumulation rate approached, but did not exceed, the theoretical quantitative maximum where one anion entering the cell corresponded to one electron leaving in respiration, and one cation entering the cell corresponded to one hydrogen ion. The quantitative relation in roots was examined by Lundegårdh (78); accumulation rate was always lower than the maximum, interpreted as being due to a considerable portion of the salt respiration (one half to one third) being concerned with the movement of metabolic anions, e.g., organic acid anions, produced within the cell; thus, in the ordinary root tissue transferred from water to 0.05 *M* chloride or nitrate, about one quarter of the anion respiration was concerned with active anion accumulation, about one quarter with internal transport of salt anions and about one half with the movement of metabolic anions within the cell. The difference from the results of Robertson & Wilkins may

¹ Wanner (139a, b) has shown that the cation absorption has a low temperature coefficient while the anion absorption has a high coefficient.

be explained on the basis that storage tissue is accumulating within the individual cells and not engaging in transport across tissue, and further that the individual cells probably leak less than the individual cells in roots.

The identity of the respiratory system with cytochrome oxidase is indicated by experiments with carbon monoxide. It has been shown in wheat roots by Sutter (128) and in carrot tissue by Weeks & Robertson (140) that the anion respiration and salt accumulation are both inhibited by carbon monoxide and the inhibition is reversed by light.

Stenlid (121) used methylene blue and α,α' -dipyridyl to study respiration of roots in salt. Dipyridyl in low concentrations inhibited 60 per cent of the oxygen consumption and almost 100 per cent of absorption of chloride and glucose. Stenlid interpreted the effect of dipyridyl in the same way as he had previously (120) interpreted the effect of orthophenanthroline, as removing a heavy metal from the respiratory enzymes. The work of Ruben *et al.* (113a) however, suggests that these substances would not remove the iron from porphyrin compounds. Methylene blue in low concentration (10^{-4} M) inhibits absorption of glucose and chloride, without inhibiting oxygen consumption.

How the application of a neutral salt to the external surface of the cell increases cytochrome oxidase activity is still unknown. It is now almost certain that the oxidase is associated with particles in the cell [Green, Loomis & Auerbach (54), Hogeboom, Schneider & Pallade (56), DuBuy, Woods & Lackey (46)]. The presence of various substances in solution has been shown by Keilin & Hartree (66) to have marked effects on the activity of succinoxidase. Chin (35) has shown that aeration of resting cells of brewers yeast produces changes in the cytochrome components and in respiration in short periods, at the expense of other components of the same system.

Lundegårdh (80) carried out a study of the inhibitory effects of cyanide and azide on the anion respiration. It was shown that the inhibition was reversed after some time when air was passed through the solution around the roots. Lundegårdh does not believe that most of the reversal might be due to the gradual removal of both cyanide and azide by the continuous air stream, and attributes it to some "adaptation" of the cytochrome system to the inhibitor. Additions of fresh solution containing the original concentrations of salt and inhibitor did not bring about the same degree of inhibition. One interesting quantitative point is that the ratio of anions accumulated to oxygen absorbed is approximately the same in the presence and absence of inhibitor, consistent with the interpretation that the oxygen uptake is associated with the liberation of a certain number of charges which exchange with ions from the external solution.

How far the cytochrome system with its separation of hydrogen ions and electrons can be regarded as the sole driving force of the accumulatory mechanism remains to be investigated. Hoagland (55), Steward (122), and Nance (97) have suggested that phosphorylations may be concerned in the provision of energy for accumulation. Nance speculated on the possibility of phosphorylations being concerned because he observed that the accumula-

tion of nitrate and chloride in excised wheat roots was inhibited by 2,4-dichlorophenoxyacetic acid, while oxygen consumption remained high. Nance suggested that 2,4-D might inhibit transfer of energy from the oxidations to the accumulatory process. The hypothesis that 2,4-D interferes with phosphorylations is not yet established but would account for a number of the effects of 2,4-D on respiratory activity and carbohydrate metabolism.

The possibility that phosphorylations are concerned in accumulation has been investigated by Robertson, Wilkins & Weeks (110) using 2,4-dinitrophenol which has been shown [Loomis & Lipmann (70), Teply (129)] to prevent the transfer of energy-rich phosphate from respiration to the phosphate transfer systems, such as adenosinetriphosphate. With carrot tissue, dinitrophenol inhibits accumulation while, at the same time, the respiration is unaffected or even increased. This respiration remains sensitive to carbon monoxide and is therefore mediated by the cytochrome oxidase system. It is tempting to speculate that the energy for the accumulation mechanism is provided by the phosphate transfer system, but these results are open to another interpretation which will be discussed in a later section.

The process of hydrochloric acid secretion by gastric mucosae, which has much in common with the process of accumulation by plant cells, has been investigated in recent years by Davies and co-workers at Sheffield. Hydrogen ions, in very high concentration compared with the concentration in the blood, are secreted into the lumen of the stomach, and chloride ions approximately equal in concentration to the hydrogen ions are secreted in about one and a half times the concentration of chloride in the blood. The process has been shown to depend upon a stimulated respiration which is sensitive to cyanide. The secretion is about three times, however, the number of hydrogen ions liberated at the cytochrome system [Crane & Davies, (41)]. Davies & Ogston (44) suggest a mechanism using the energy of unstable phosphate esters to transport the hydrogen ions. A mechanism of this type dependent on phosphorylation has been discussed by Robertson (107) as a possible additional mechanism for accumulation in plant cells, but so far there is no evidence for its participation.

The theoretical mechanisms discussed by Franck & Mayer (51) would allow several different possibilities for accumulation. One observation not satisfactorily reconciled with the theories of accumulation is the effect of light which, in both *Nitella* and *Vallisneria* [Arisz (5)], influences the rate of accumulation. Arisz concludes that the influence is not indirect through the products of photosynthesis, but is direct, affecting processes in the cytoplasm. Steward & Street (123), observing that accumulation frequently accompanied protein synthesis in growing cells, suggest that phosphorylated energy-rich nitrogen compounds formed with metabolic energy at the outer surface of the cytoplasm might possess amphoteric properties, and might thus accompany both anions and cations across the cytoplasm. Steward's view differs from that of Lundegårdh and others in associating the driving force more closely with protein synthesis. It is perhaps too early to decide

whether the driving force is simply the separation of ions in the late stages of respiration or whether phosphorylated compounds with or without nitrogen are also concerned. This involves the carrier system, to be discussed in the next section.

The carrier system.—No carrier in any active transport mechanism has been identified, though there is evidence for some substance which combines with potassium. Thus, Eddy and co-workers (47) have shown that *Bact. lactis aerogenes* takes up radioactive potassium which could not be replaced by sodium; lithium and caesium had small effects; rubidium replaced the potassium with about one quarter efficiency. Potassium appeared to be an enzyme activator in competition with hydrogen ions for the active enzyme centres where it was effective, but the nature of the substance with which it combined was unknown.

Cowie, Roberts & Roberts (40) and Roberts, Roberts & Cowie (104), using *E. coli*, have provided some clues to link the accumulation of potassium with (a) carbohydrate metabolism, (b) phosphate metabolism, and (c) substances which can discriminate between sodium and potassium. The applicability of their conclusions to other cells may have considerable importance. Using Na^{24} , they found that *E. coli* was permeable to sodium which was easily washed from the cells suggesting that it was not bound. In contrast, potassium (K^{42}) was markedly concentrated; in addition, there was freely diffusible potassium in equilibrium with the medium, and the cells were highly permeable to potassium ions. Without metabolism, the potassium compounds disintegrated slowly and ionic potassium was released. Following the addition of glucose, a large uptake of potassium occurred which could be limited by the glucose or potassium available.

Attempts to isolate the potassium compounds formed either during growth or with glucose metabolism were unsuccessful. It appeared that the compounds were extremely unstable and dissociated as soon as they were removed from the cells. Other carbohydrates and metabolic intermediates were investigated. The initial uptake of potassium when glucose-1-phosphate was added exceeded that when glucose was added. This is interpreted as being due to the potassium combining with hexose phosphates or other phosphorylated intermediates. Sodium showed no evidence of combination; rubidium behaved like and competed with potassium. Potassium was taken up and bound before any appreciable binding of phosphate, added simultaneously, occurred.

These authors believe that, after diffusion into the cell, the potassium fixation occurred on a compound formed by the hexose with some substance already in the cell. Approximately two atoms of phosphorus were bound per molecule of glucose. They regard the hexoses as forming potassium salts after phosphorylation by adenosinetriphosphate. It was not possible to decide which of the hexose phosphates was important, though the data suggested that glucose-6-phosphate and fructose-6-phosphate might have been specially important. They conclude that the reactions are too rapid, too

specific and too closely and quantitatively linked to carbohydrate metabolism to be explained in terms of protein or lipid binding of the potassium.

No similar attempts seem to have been made to look for specific substances responsible for the transport of ions into the cells of higher plants, though the suggestion has been made that carrier substances are produced metabolically [Lungedårdh (72, 73), Overstreet & Jacobson (100), Jacobson & Overstreet (63)]. In recent work, Jacobson, Overstreet, King & Handley (64) have used barley roots to investigate the cells' capacity for binding potassium as a guide to the carrier of cations, on the hypothesis that the binding would be related to the combining power of a carrier substance for potassium and for hydrogen ions according to the reaction:—



where R is the carrier. Large quantities of potassium were lost from roots in solutions of hydrochloric acid at lower pH values and only small quantities at higher values. These losses at a given pH could be offset and even reversed by appropriate increase in the potassium chloride in the external solution. The substance combining with the cations was thought to be a product of metabolism but not an organic acid and seemingly not specific, though calcium played a special role and did not compete for this carrier of potassium.

It is doubtful whether this work adds to our knowledge of the uptake of cations. Since many substances in the cytoplasm—proteins, amino acids and organic acids—are weak electrolytes, changes in pH will affect the number of ionised particles. This, of course, affects the organisation of the cytoplasm profoundly, as shown by the loss of cell contents at lower pH values. These changes will almost certainly produce effects which will mask any specific substances concerned with transport. No specific compounds which would act as carriers of the monovalent or divalent cations have been identified in higher plants. Carriers of anions such as the halides, which are readily accumulated, are also unknown. The possibility that such carriers occur in nonaqueous phases such as in cytoplasmic particles must be considered. It is desirable, therefore, to examine the physical structure of the cytoplasm to determine what sort of medium this offers for an accumulation process.

The sub-microscopic structure of the cytoplasm.—Whatever the carriers involved in the active transport system, their presence and activity must be related to the sub-microscopic architecture of the cytoplasm of the plant cell. Ions entering the surfaces of cells must be transported by the carriers to the vacuole from which they can escape only very slowly, i.e., the ions now free in solution have been transported across a region of high resistance to diffusion. Some workers [e.g., Holm-Jensen, Krogh & Wartiovaara (57)] have located this resistance at the outer surface of the cytoplasm, some in the whole cytoplasmic region and some at the inner surface of the cytoplasm (tonoplast); the latter view has most to commend it.

Arisz (5, 6, 7) has shown that, in leaves of *Vallisneria*, ions penetrated the outer surface of the cytoplasm and here might be transported by the accumulation mechanism to the vacuole where they were free in solution and caused a considerable increase in the osmotic pressure. Ions present in the vacuole diffused outwards in appreciable quantity only when the cytoplasm was injured. Injury also inhibited the movement of ions by the active transport mechanism.

The problem has been discussed by Lundegårdh (81) recently; his theory of accumulation postulates that the single members of the cytochrome system are attached to an organized structure to form an electron ladder. Anions are moved in a direction opposite to the electrons, and cations accumulate against the hydrogen ions which are escaping simultaneously. Lundegårdh considers that though the location of the cytochrome-cytochrome oxidase system in the cell is still undecided, its firm attachment to the protoplasmic structure has been established. If the electron ladders are built perpendicularly to the surface of a membrane separating two levels of accumulation (e.g., the cytoplasm and the medium, or the cytoplasm and the cell sap), salts will be actively accumulated provided that the oxygen comes from the outside and the hydrogen from the inside. A polar transport would also occur as the statistical result of an irregular distribution of particles between two oxidation levels. He concludes that as oxygen concentration limits the rate of accumulation only at very low tensions, the structural polarity of the enzyme system itself is probably the more important.

It can be shown that in the cell there is a considerable resistance to back diffusion which is not destroyed when the accumulatory mechanism is inhibited [Robertson (105)]. As mentioned earlier, either an orientated lipid or lipo-protein membrane, or the presence of a number of indiffusible ions of one charge would result in a high resistance to movement of ions. A lipid membrane probably exists at the tonoplast; such membranes have been discussed by Davson & Danielli (45). The effects of indiffusible ions on movement of electrolytes through a tissue have been considered by Briggs & Robertson (20), Frey-Wyssling (52), and Eriksson (50).

Since the cytoplasm is penetrated readily by ions of a positive charge and since the predominant ions of the normal cytoplasm are anions of weak electrolytes, it is influenced by the hydrogen ion concentration of the external solution, a fact which is often neglected in discussions of effects of pH on entry of weak electrolytes. Aleshin & Yastrebov (3), following earlier work of Lundegårdh (72), have examined the potential at root surfaces and the effects of changes in pH of the medium on the pH of roots of wheat seedlings. The relative uptake of anions and cations was affected by the root potential, and both ions were taken up at equal rates only at the iso-electric point of the protoplasmic proteins. With carrot tissue [Robertson & Wilkins (111)], the entry of anions from the external solution increases markedly as the external solution is reduced from pH 7 to pH 4, presumably due to the suppres-

sion of the cytoplasmic anions which, from consideration of the Donnan effect, allows the penetration of more of the mobile anions from the external solution.

After accumulation, most of the resistance to the leakage of ions is probably in the vacuolar membrane, a conclusion which receives considerable support from the work of Arisz on the uptake and transport of chloride by the parenchyma of leaves of *Vallisneria*. The well known observation that the addition of a metabolite to the cell surface produces large effects, even though the vacuolar concentration of the same metabolite may be high, suggests that the vacuolar membrane has a high resistance, and further, since the resistance to non-electrolytes is high, that it is mostly due to lipids. In hypotheses of accumulation, therefore, we must allow for the entry of ions into the vacuole through a high resistance region which is probably lipoidal. At present we do not know how thick this region of lipid concentration may be.

Lundegårdh (81) has pointed out that the disposition of the cytochrome oxidase system in plant cells has not been determined. There are, however, some very valuable indications of its probable position. First, it has been well defined in animal cells. From the work of a number of investigators, particularly that of Green, Loomis & Auerbach (54) and of Hogeboom, Schneider & Pallade (56), it has become clear that, not only is the succinoxidase enzyme system associated with the mitochondrial particle, but so also are all the reactions of the Krebs acid cycle, including the associated phosphorylations. It has been shown that the cytochrome components of the respiratory system are carried on the mitochondria of *Nicotiana* and *Lonicera* [DuBuy, Woods & Lackey (46)]. The possibility arises, therefore, that the mitochondria or microsomes act, not only as the sites of energy release for accumulation, but also as the carriers of the ions. The advantage of such a system would be clear; the mitochondria are lipoprotein complexes with about 40 per cent lipid [Ball & Cooper (10)]. One effective way of putting ions through a lipid region would be to enclose them in a mobile particle predominately lipid; mitochondria have the requisite mobility since they move with protoplasmic streaming. Such a hypothesis will prove of value in co-ordinating some of the earlier observations; Mullins (95) showed that radioactive ions (potassium and phosphate) supplied to the cells of *Nitella* became concentrated on granules in the cytoplasm; Arisz (4) suggested that the transport of ions through the cytoplasm is on microscopic particles and further that the streaming is affected by salts, though in his subsequent work (7) with *Vallisneria* leaves he was not able to demonstrate a connection between vacuolar accumulation and visible streaming. Further, the functioning of mitochondria in the transport could be reconciled with the apparent importance of phosphate transfers in the accumulatory process [Robertson, Wilkins & Weeks (110)]. It has been shown that the transfer of energy-rich phosphates, which is interfered with by dinitrophenol in the cyclophorase system, is essential to the maintenance of the particle [Teply (129), Still (124)]. The particles may be disorganised in the presence of dinitrophenol and

this would interfere with the transport of the ions, without increasing the leakage of ions already accumulated. Finally, it has frequently been noted that mitochondrial activity is intensified in secreting animal cells. During the secretion of hydrochloric acid by the gastric mucosa, mitochondrial activity is increased markedly at the onset of secretion.

TRANSPORT WITHIN THE PLANT

Across the root.—In recent years, Arisz (4) and Lundegårdh (73) have adopted the view that the root acts like a single cell in passing ions across successive layers of protoplasm. A root so functioning is sometimes called a symplast. While ions may move passively in cell walls along the concentration gradient, active transport in the living cells brings about most of the movement and results in final secretion into the xylem. Lundegårdh (74, 76, 77) extended his theory to allow for the effects of the salt accumulation process on the exudation of inorganic ions and water. He concluded that ions were accumulated in such concentration that they subsequently leaked into the non-living xylem elements, and, for osmotic reasons, carried with them the stream of absorbed water. A similar concept—the “accumulation level”—was used by Wiersum (141). Lundegårdh considered the release of water and ions into the xylem to be controlled by an anaerobic phase of the respiration. In 1949, after comparing the effects of glycolytic inhibitors with those of the aerobic stages, he concluded that the exudation was linked to glycolysis, the “third respiration” referred to earlier, which seemed to accelerate the secretion of cations and consequently of anions into the xylem. This view was modified subsequently (81) when it was shown that the exudation of salts was not linked to the anaerobic glycolysis; further, it was shown that vertical movement in the cortex was insignificant compared with that along the radius of the root and vertically in the stele. The anatomy of the root favours exudation into the stele because of the high permeability of all cells except those of the epidermis. In this connection, Burström (29) showed that ion absorption was increased in roots from which the epidermal layer had been stripped with *n*-diamylacetic acid, presumably due to the increased access of ions to the inner tissues which could still accumulate. Lundegårdh considered that exudation occurred towards the centre of the root because of the increasingly anaerobic conditions in the region enclosed by the endodermis; this requires checking, as Brown (21) and Woodford & Gregory (145) suggest that roots attached to the plant may be efficiently aerated by the intercellular space system. Van Nie, Helder & Arisz (133) also studied secretion of ions into the xylem of roots and the osmotic regulation of the exudate so formed. Their observations support the idea of the dependence of exudation upon the salt accumulation process. Neither Arisz and his collaborators nor Lundegårdh found any evidence for a metabolic transport of water as suggested by van Overbeek (134) for roots and by Bennet-Clark & Bexon (12) for storage tissue.

Broyer (23) studied transport of radioactive isotopes across the root. At

low temperatures, migration of electrolytes in high salt roots might be restricted to adsorption exchange; at higher temperatures, ion exchange was less important than metabolic accumulation and in low salt roots metabolic accumulation might be large even at low temperature. Broyer also suggests that ions move along continuous protoplasmic paths (the symplast) to the xylem, especially in high salt roots; in low salt roots, the main movement is into the vacuoles.

Various problems require more investigation. For example, it is not known whether the protoplasm continues through cell walls. Again, in spite of the importance of the organic acid cycle [Machlis (84), Lundegårdh (78)], organic anions do not appear to be secreted into the xylem; their retention in the cells may be related to the operation of the organic acid cycle in mitochondria.

Through the conducting system.—Salts liberated into the xylem elements in the root move through the plant with the transpiration stream or by diffusion, the only polarity being due to the direction of the stream or of the concentration gradient. Cells along the path absorb the ions by exchange, combination, and accumulation. Nutrients also move in the phloem; the relative importance of xylem and phloem transport undoubtedly varies with the nature of the nutrient and the conditions of supply and demand in the plant [see Biddulph (14) and Curtis & Clark (42)].

It has been known for some time that absorption of ions by roots is not closely dependent on the amount of water moving in the transpiration stream [see Hoagland (55)]. MacEwan (83) found that radiophosphorus (P^{32}) in squash plants moved from roots to leaves predominantly in the xylem. Rate of uptake by roots was independent of rate of accumulation in leaves which was primarily dependent on the transpiration rates. Similar dependence on transpiration was demonstrated with Ca^{45} . Radiophosphorus was preferentially accumulated in apical leaves.

Independence of transport and accumulation rates was also demonstrated by Hopkins, Specht & Hendricks (58) with tomato plants. Low oxygen supplies to the roots restricted ion accumulation by the roots, but did not affect transport rate through the plant.

MacVicar & Burris (85), using N^{15} supplied to the tomato plant as ammonium sulphate, observed rapid increase in concentration of labelled nitrogen in the mature tissues which suggested the conversion of the ammonium ion to organic nitrogen compounds and their transfer, though the possibility of translocation of ammonium ions was not excluded.

A special problem of absorption and movement was studied by Bledsoe, Harris & Tisdale (17) and Bledsoe & Harris (18) who showed that the peanut plant cannot absorb enough calcium (Ca^{45} was used) for normal fruit development unless the gynophores are supplied directly. On the other hand, the gynophores cannot absorb and transport enough calcium to offset deficiency symptoms in the plant when the roots are not supplied.

Mechanisms of export and movement of nutrients from one plant organ to another require further investigation. Humphries (59) has investigated

seasonal variation in wood and bark of the cacao tree. Rhodes, Templeman & Thruston (103) have shown that the growth regulator 4-chloro, 2-methylphenoxyacetic acid results in lower concentrations of potassium than usual in the tops of rape and tomato plants. Moore (92) found downward translocation of phosphorus (P^{32}) in maize plants, only after it had been moved into the shoots. Some slight loss from the roots occurred in a phosphate deficient medium.

Biddulph (14), with radioactive ions supplied both in nutrient solutions and by injection, showed that both iron and phosphorus were exported from mature leaves. With phosphorus, which moved in the phloem, a diurnal trend in movement was apparent, with more rapid movement in the daylight. This re-export from leaves may be inferred to have had an effect on uptake, but most of the phosphate passed to the roots was recirculated in the xylem. In a further study of P^{32} , S^{35} , Fe^{55} and Ca^{45} , Biddulph (15) showed that while phosphorus was freely mobile, sulphur was freely mobile at first but was readily incorporated into organic compounds, iron was readily mobile under some conditions, and calcium (like strontium and barium) was freely mobile in the phloem under conditions of low calcium in the tissues, and low phosphorus and low pH in the injected solution (16).

The salt content of the xylem sap suggested by Burström & Krogh (30, 31) as the main medium of transport to buds, is too low to account for the amounts taken up, unless the sap is assumed to circulate. It is possible that the solutes reach the buds via the phloem where their concentration may be greater than in the xylem [Burström (26)]. Bleeding, which is absent in some trees with rapidly growing buds, appears to have no essential connection with the movement of nutrients.

It must be concluded that the problem of the mechanism of nutrient movement within the plant has still to be solved. The recent discussion by Curtis & Clark (42) adds no new facts or theories. The mechanism of movement of inorganic constituents in the phloem is as obscure as that of carbohydrate transport.

CONCLUSION

Since future work will be directed to filling the gaps in our knowledge of absorption and transport, some outstanding problems can be indicated.

While the theory of the Donnan effects resulting from cells in electrolyte solutions is well developed, we are ignorant of the nature and concentrations of the substances on which ion exchange occurs. We need more exact information before quantitative treatment is possible; for the same reason, we are unable to distinguish clearly between resistance to diffusion due to immobile anions in the cytoplasm and resistance due to lipid membranes.

Except for the metabolites which combine with certain nutrients, substances which form compounds with nutrient ions are unknown. Recent interest in chelation chemistry has opened the way for a study of the affinities of cell substances for different inorganic ions; such investigations will con-

tribute to the understanding, not only of mechanism and transport, but also of nutrient function.

The combination of potassium with some cellular substance, which apparently does not combine with sodium, remains one of the outstanding problems of biology. Suggestions, culminating in the observations on *E. coli*, that phosphorylated compounds are involved, open a profitable field for investigation. On theoretical grounds it is difficult to picture substances which in an aqueous medium combine with potassium and have a low affinity for sodium; the possibility of cations combining temporarily with substances in non-aqueous regions of micro-proportions (such as lipo-protein complexes) requires investigation.

Accumulation in cells has been shown to be due to active transport across the cytoplasm to the vacuole where the ions are free in solution. The driving force is probably associated with the respiration at a stage where charges are separated into hydrogen ions and electrons. Phosphorylations have been shown to be related to the accumulation mechanism, but not necessarily directly as the energy-providing steps—they might be necessary to the maintenance of sub-microscopic structures which make accumulation possible. Knowledge of the sub-microscopic morphology of the cytoplasm is essential to complete a hypothesis of the accumulation mechanism, particularly as the steps in respiration probably concerned with accumulation have been shown to be associated with protoplasmic granules.

Beyond the fact that ions are moved across the root by a mechanism dependent on accumulation, and liberated into the xylem, where they can move with the diffusion gradient or with the transpiration stream, we know little of movements of nutrients in the plant. Ions can move in the phloem; this path is probably important in the remobilisation of nutrients which occurs in the plant, but we are ignorant of the mechanism.

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THE ROLE OF THE MINERAL ELEMENTS IN PLANT NUTRITION

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At the suggestion of the Editor, special attention has been given here to the relation of mineral nutrients to enzyme systems and to a general discussion of some less familiar aspects of nutrient interrelationships, including where relevant, studies of microorganisms and occasionally animal metabolism.

POTASSIUM, AMMONIUM, CALCIUM, AND CHLORINE

Little has come to light recently on the still elusive role of potassium. Evidence obtained by Stout, Overstreet, Jacobson & Ulrich (1) from studies with radioactive tracers was extended by Olsen (2, 3) with beech leaves to show that contrary to accepted ideas, up to 30 per cent of the total potassium may be present in a bound form, possibly adsorbed to a protein fraction (as in a base exchange system) from which it may be eluted by 0.1 *N* CaCl₂ or great excess of water (3).

The reviewer is of the opinion that potassium, and to some extent calcium in complementary fashion, maintain cell organisation, hydration and permeability and hence directly or indirectly influence many enzyme systems, e.g., the condensation or hydrolysis systems inferred by Cooil & Slattery (4) and already reviewed by Mulder (5). The accumulation of free amino acids reported by Steinberg, Bowling & McMurtrey (6, 7) in potassium-starved tobacco also suggests breakdown of protoplasmic organisation. A few instances, however, are known where direct activation may be inferred. Muntz (8) found that in yeast, phosphorylation of hexose monophosphate to the diphosphate depended on potassium, which was not required for subsequent fermentation or for formation of the monophosphate. Ammonium could replace potassium in the system. Lardy & Ziegler (9) found that phosphorylation of pyruvic acid by adenosinetriphosphate (ATP) in rat muscle also required potassium, but phosphorylation of creatine did not. Evidently the need of potassium in phosphorylation is not general.

An important effect of potassium may be its ability to maintain limited iron supplies functional for chlorophyll formation. Instances in earlier work were noted by Wallace & Hewitt (10) and Jones & Hewitt (11). The latter, in a detailed study of the problem (11), and Jones (unpublished) have shown that potassium deficiency causes acute chlorosis of young leaves of potato plants when iron is lacking, but adequate potassium permits chlorophyll formation; shoots are then initially greener than old leaves. This phenomenon is associated with reduced phosphorus uptake, confirmed by Wallace & Bear (12), but phosphorus deficiency does not occur and potassium does not antagonise iron (11). It seems that the utilisation of limited iron supplies is

more efficient with adequate potassium. A reciprocal effect in which high iron levels cause temporary retention of potassium in older leaves when it would normally be translocated to younger parts under deficiency conditions is also inferred from visual and analytical data (11). The relation of this effect to "bound" potassium is of interest. The earlier work and ideas on the role of potassium are summarised by Cooil & Slattery (4).

Calcium is apparently a direct activator (*in vitro*) for certain phosphatase enzymes identified by Kalckar (13) and Krishnan (13a) in potato tubers. These enzymes catalysed both the dismutation of 2 molecules of adenosine-diphosphate (ADP) to give ATP and adenylic acid and the removal of two phosphate radicles from ATP to give adenylic acid. Their biological significance is obscure.

No satisfactory evidence for the essential nature of chlorine is forthcoming for higher plants, and Arnon & Whatley (14) recently disposed of the possibility of this element being needed for photosynthesis *in vivo*.

MAGNESIUM

The presence of magnesium as a constituent of chlorophyll presents interesting features. It apparently enters into the molecule prior to the final appearance of chlorophyll as Granick (15) has shown that a magnesium protoporphyrin is a possible precursor of chlorophyll in *Chlorella*. Granick (16) had previously found that an x-ray mutant accumulated protoporphyrin 9 instead of chlorophyll. The "chlorophyll" of *Laminaria* was also found by Granick (17) to be a magnesium derivative possibly of phaeoporphyrin in which the metal was more firmly bound and the phytol residue was lacking.

An important general function of magnesium is the activation of enzymes containing sulphydryl groups ($-SH$) especially those involving phosphorus metabolism. In many instances results relate to animal and yeast material but it is probable that in general they will apply to higher plants. Dixon [(18) p. 43] states that all phosphokinases depend on $-SH$ groups and are activated by magnesium. Important examples are the hexokinase of yeast described by Bailey & Webb (19) and Berger, Stein, Colowick & Cori (20), (although the latter could detect no influence of $-SH$ groups on the activity of the enzyme) and the phosphokinase of pigeon liver studied independently by Dixon (18) and by Speck (21). This enzyme causes the phosphorylation of glutamic acid by ATP prior to the formation of glutamine. Bailey & Webb (22) concluded that yeast pyrophosphatase is also a sulphydryl enzyme and magnesium is a specific activator. In some phosphorase enzymes other metals, particularly manganese, may replace magnesium. Examples include the yeast phosphoglucomutase which, according to Cori, Colowick & Cori (23), is similar to that found in rabbit muscle and is activated equally by magnesium, manganese, and cobalt, and the enolase of *Escherichia coli* studied by Utter & Werkman (24).

Fleisher (25) found that magnesium deficiency in *Chlorella* limited the rate of photosynthesis long before there was appreciable reduction in chloro-

phyll content. A role in a photosynthetic phosphorus enzyme system was suggested.

Webb (26) observed that magnesium-deficient cultures of *Clostridium welchii* failed to undergo normal cell division and became filamentous. This result may be further evidence of the activation and role of -SH groups in cell division and complementary to the independent work of Nickerson and others on cobalt discussed below.

According to Bernhauer, Iglauer, Knobloch & Zippelius (27), *Aspergillus niger* requires magnesium for citric acid production, and a role in the citric acid cycle may be implied. Krampitz & Werkman (28) found that magnesium or manganese alone could activate systems causing β -decarboxylation of keto acids in *Micrococcus lysodeikticus* but that co-carboxylase was needed as well to achieve α -decarboxylation. Soya bean carboxylase was found by Mee (29) to be activated by magnesium possibly more efficiently than by manganese.

Other enzymes where magnesium is one of two or more alternative activating metals which usually include manganese are a number of dehydrogenases, e.g., isocitric dehydrogenase, carboxylases, and peptidases dealt with below.

PHOSPHORUS

Phosphorus plays a fundamental role in the breakdown of glucose by transfer of energy in graded steps, and in the utilisation of this energy for synthetic reactions. The enzymes which are concerned in the transfer of phosphate and the formation of various phosphate bonds have been termed phosphorases by Dixon (18) and classified into six groups as follows: (a) bond energising enzymes controlling the formation of energy-rich phosphate bonds, e.g., triose phosphate dehydrogenase, (b) phosphokinases (e.g., hexokinase) which depend on ADP for transfer of the phosphate group from one molecule to another without the intermediate formation of free phosphate, (c) phosphotransferases which transfer phosphate ester groups direct without ADP and are not concerned with energy-rich bonds, (d) phosphomutases (e.g., phosphoglucomutase) which transfer phosphate from one position to another in the same molecule, (e) phosphorylases which catalyse synthetic reactions with the elimination of phosphate bonds (e.g., the synthesis of starch from glucose-1-phosphate) and (f) phosphatases (e.g., yeast pyrophosphatase), which split off inorganic phosphate.

Key materials in the transfer of energy rich phosphate bonds are ADP and ATP. The latter is formed from ADP by addition of an energy-rich phosphate bond, a concept developed by Lipmann (30). Dixon [(18) p. 45] has likened the role of ADP in the transfer of phosphate groups to that of the coenzymes I & II (DPN & TPN) in the transfer of hydrogen. ADP (and the ATP produced) can link phosphate bond energy-transferring enzymes in the same manner as many dehydrogenase systems are linked by the phosphorus containing coenzymes I & II. Dixon [(18) p. 20] points out

how the energy requirements of the cell (in terms of utilisation of ATP or an ATP reservoir) control the forward reactions of glycolysis by the value of the ADP/ATP ratio.

Dixon [(18) p. 44] illustrates the role of ATP as a source of synthetic energy by the synthesis of glutamine from glutamic acid and ammonia, the energy being obtained from liberation of phosphate originally conferred on the glutamic acid by energy-rich ATP. The probability that peptide synthesis is also energised by the energy-rich phosphate bond of a phosphorylase was indicated by Cohen (31), and Eaton (31a) found that amide nitrogen accumulated in phosphorus-deficient soybeans. The problems of phosphate bond energy transfer are discussed by Kalckar (32) and Lipmann (30). Dixon (18) has shown that simple rules can be devised to predict the effects of pH and percentage phosphorylation on the energy value of phosphate bonds (the μ P value) and on the direction of phosphorylation in different systems.

The reversible oxidative decarboxylation of malic acid in plants (and probably an essential link in the tricarboxylic acid cycle) depends, according to Conn, Vennesland & Kraemer (33) specifically on the presence of TPN (coenzyme II). They concluded that no β -decarboxylase yet investigated was independent of this malate enzyme. Although widely distributed, it was often difficult to detect for the curious reason that another enzyme caused destruction of the TPN. This could be prevented by the addition of ATP and provides the example of where one phosphorus compound maintains the existence (presumably by a phosphokinase system) of another essential catalytic phosphorus-containing material. Kornberg (34) obtained evidence in autolysed yeast of a possibly analogous system in which coenzyme II was synthesised from coenzyme I and TPN. Manganese or magnesium were required for activation.

Another important phosphorus compound recently identified is pyridoxyl phosphate. This is regarded as an essential coenzyme for transamination by animal and bacterial enzymes according to Lichstein, Gunsalus & Umbreit (35), Green, Leloir & Nocito (36) and O'Kane & Gunsalus (37), and for amino acid decarboxylation according to Baddiley & Gale (38) and Schales, Mims & Schales (39) who found that glutamic acid decarboxylase was widely distributed in plants. Pyridoxyl phosphate was found by Wood, Gunsalus & Umbreit (40) to be needed for the conversion of tryptophane to indole and pyruvic acid by *E. coli*, and for the synthesis of tryptophane from serine and indole according to Umbreit, Wood & Gunsalus (41).

It was suggested by Fleisher (25) and Ruben (42) that phosphate compounds are concerned in photosynthetic reactions and the idea was elaborated by Emerson, Stauffer & Umbreit (43). Benson *et al.* (44) have now identified phosphoglyceric acid as an early product in photosynthesis. The problem whether respiration and photosynthesis proceed in the same cell by similar reactions in opposite directions simultaneously might be resolved if the synthetic reactions proceed under one set of phosphate enzymes whilst the catabolic reactions are handled by a different set. In this context it is interesting to speculate whether the magnesium chlorophyll complex may be a co-

enzyme for activation of a phosphate enzyme system closely associated with chloroplasts and able to convert the energy absorbed by the chlorophyll into energy-rich phosphate bonds.

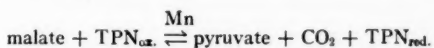
MANGANESE

Much that applies to the discussion of magnesium or phosphorus is relevant to manganese also. This element may influence dehydrogenation, oxidative and non-oxidative decarboxylation, peptide hydrolysis, arginase, nitrogen metabolism, and iron metabolism.

Organic acid metabolism.—Manganese-activated dehydrogenase systems known to occur in plants include the widely distributed isocitric dehydrogenase system isolated by Adler, von Euler, Gunther & Plass (45) and von Euler, Adler, Gunther & Elliot (46) and more recently found in oat coleoptile by Berger & Avery (47). The "hexose dehydrogenase" reported by Rudra (48, 49) in *Phaseolus mungo* was thought (on slender evidence) to catalyse the synthesis of ascorbic acid. The effects of manganese on ascorbic acid content of plants are not consistent, but Harmer & Sherman (50) found a marked depletion in foliage of many manganese deficient plants.

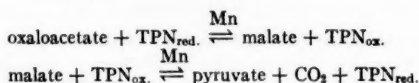
The isocitric system (45, 46) is activated by magnesium as well as manganese but less efficiently and at a higher optimum concentration ($2.5 \times 10^{-3} M$ compared with $5 \times 10^{-4} M$). Levels above the optima for either metal cause a sharp decline in activation. Ochoa (51, 52) concluded from a study of animal tissues that the conversion of isocitric to α -ketoglutaric acid and carbon dioxide is the result of two distinct enzymatic reactions: (a) dehydrogenation, involving TPN (coenzyme II), to oxalosuccinic acid which is independent of manganese (or magnesium), and (b) decarboxylation of oxalosuccinic to α -ketoglutaric acid, which requires the metal. This is contrary to Adler *et al.* (45) who considered the first step to be metal activated.

The freely reversible conversion of malic to pyruvic acid in parsnip and parsley roots was studied by Vennesland, Gollub & Speck (53). They concluded that it follows a course analogous to isocitrate: dehydrogenation of malate to oxaloacetate followed by a decarboxylation of the oxaloacetate to pyruvate. Requirement of manganese for the first step was not shown independently but the two distinct systems were not separated. The optimum concentration *in vitro* was $0.01 M$ $MnCl_2$. In addition, however, to the two-step decarboxylation (activated by manganese) of malate by way of oxaloacetate, evidence was also obtained that plants contain a "malic enzyme" (33) analogous to that found in pigeon liver (54). This "malic enzyme" was identified in wheat germ and beet, carrot and parsnip root, leaf and seed. In the presence of manganese the following TPN-linked reversible oxidative decarboxylation takes place:



Conn, Vennesland & Kraemer (33) found that cobalt could replace manganese in this widely distributed and important enzyme system.

Manganese also activates a reversible oxaloacetic decarboxylase system in pigeon liver where two oxidation-reduction steps have the net effect of nonoxidative decarboxylation. The reactions identified by Veiga-Salles, Harary, Barfi & Ochoa (54) are:



Here both steps are manganese-activated and the overall reaction is catalysed by malic acid. The points of distinction between these systems are noteworthy and illustrate the versatility of these manganese-activated decarboxylations. The so-called pyruvic dehydrogenase of *Bacillus coli* studied by Still (55) was activated by manganese or magnesium and less efficiently by cobalt.

Many decarboxylations are nonoxidative and may also involve manganese. Vennesland, Ceithaml & Gollub (56) found that manganese was needed for the reversible reaction between α -ketoglutaric acid and carbon dioxide to produce oxalosuccinic acid. This they regarded as catalysed by a distinct enzyme system in parsley root, able to bring about carbon dioxide fixation. This reaction is the reverse of the second step in the isocitric dehydrogenase system (45, 46). Ceithaml & Vennesland (57), however, found that cobalt could also activate the parsley root enzyme *in vitro* as in the malic oxidative decarboxylase above (33). Vennesland & Felsner (58) and Gollub & Vennesland (59) indentified also an oxaloacetate decarboxylase in parsley root which was specifically activated by manganese (and not by magnesium). Vennesland (60) found this enzyme was widely distributed in the organs of all 13 species of plants investigated. According to Speck (61), however, many cations could activate the parsley and radish root enzymes including $\text{Mn}^{++} > \text{Co}^{++}, \text{Cd}^{++} > \text{Zn}^{++} > \text{Ni}^{++}, \text{Fe}^{++}, \text{Mg}^{++}$ in order of activity. Herbert (62) obtained a pure preparation of the oxaloacetic decarboxylase from *M. lysodeikticus* studied by Krampitz & Werkman (28) and also found that $\text{Cd}^{++}, \text{Co}^{++}, \text{Ni}^{++}$ were able to activate the enzyme in addition to Mn^{++} or Mg^{++} , but trivalent metals were inactive. A pyruvic decarboxylase found by Vennesland & Felsner (58) in parsley root was independent of manganese, but according to Kalnitsky & Werkman (63), the pyruvic decarboxylase of *E. coli* depended on manganese (or magnesium) as well as on cocarboxylase. These authors (64) found an oxaloacetic decarboxylase that required only manganese, which might be replaced by cobalt in this organism, according to Speck (61). Von Euler, Adler, Gunther & Vestin (65) found that the pyruvic decarboxylase of yeast was activated by manganese and cocarboxylase. Plaut & Lardy (66) studied an oxaloacetic decarboxylase in *Azotobacter vinelandii*. This enzyme system was activated by manganese, cobalt, or zinc and to a slight extent by magnesium but not by nickel or iron.

Vennesland (67) found that manganese, metmyoglobin, and oxygen caused nonenzymatic oxidative decarboxylation of oxaloacetic acid, but the

product was malonic acid which inhibits the succinic dehydrogenase system. In most of such nonenzymatic decarboxylations, however, manganese (and magnesium) possess relatively low activity compared with several others, including trivalent metals, according to Krebs (68), Speck (61), and Ochoa & Weisz-Tabori (69).

It is clear that manganese (sometimes replaceable by magnesium or other metals) is important in the reversible decarboxylase and associated dehydrogenase systems involving the organic acids. Stern, Shapiro & Ochoa (70) and Korkes, Stern, Gunsalus & Ochoa (71) have now elucidated mechanisms in yeast and other microorganisms whereby citric acid is synthesised from oxaloacetic and pyruvic or acetic acids, in the presence of phosphate and either manganese or magnesium. The key role of these metals in the full operation of the tricarboxylic acid cycle thus comes clearly into perspective. The observations of Lundegårdh (72) contending that manganese stimulated respiration and carbon dioxide output by wheat roots, and those of Arnon (73) that manganese (as well as copper) reduced the need for aeration for barley when supplied with ammonium nitrogen may be related to this conclusion.

Peptidases and arginase.—Manganese is one of several metals that activate peptidase enzymes. Peptidases identified in malt, spinach, and cabbage by Berger & Johnson (74, 75) hydrolysed leucyl-glycine and were activated by manganese and less effectively by magnesium. Bamann & Schimke (76, 77) also found that *d*- and *l*-leucyl-glycine peptidases obtained from oat, barley, asparagus, and several seeds were activated by manganese, but hardly at all by magnesium and cobalt. Berger & Johnson (74) found that whereas the leucyl-glycine peptidase of *Proteus vulgaris* was activated by either manganese or magnesium, the di- and alanyl-glycine peptidases of *Phytomonas tumefaciens* were activated by manganese only and in *B. megatherium* by cobalt also. Diglycine peptidases in yeast and malt were activated by manganese or cobalt but pH effects modified their relative efficiency, cobalt being ineffective above pH 8.5 and manganese being ineffective below pH 7. The activation of dipeptidases in yeast and *B. megatherium* was found by Berger & Johnson (78) to be more complex, as either cysteine or manganese produced activation, which was much greater with both together. Iron or glutathione replaced them at lower efficiency.

Schales & Roux (78a) similarly found that either iron or dihydroxyphenyl-alanine (DOPA) activated a dipeptidase in yeast. Maschmann (79) also found that peptidases in several bacterial species were activated by cysteine and either iron or manganese with different efficiency, usually greater with iron.

Arginase in Jack bean was studied by Stock, Perkins & Hellerman (80) and by Anderson (81); manganese was one of several metals that activated the enzyme. Others included cobalt, ferrous iron, and nickel. According to Anderson (81), activation by iron was weak unless cysteine was present when it became equal to that with cobalt. Hellerman & Perkins (82) found that urease obtained from Jack bean also hydrolysed arginine in the presence of

manganese, cobalt, nickel or iron. Edlbacher & Baur (83) found that dialysed yeast arginase was reactivated by manganese, slightly by cobalt, but scarcely at all by other divalent ions.

Nitrate assimilation.—The relation of manganese to nitrate assimilation has been dealt with by Mulder (5), but a few points merit comment. Mulder concluded that the observations of Hewitt, Jones & Williams (84), namely, that manganese deficiency resulted in accumulation of certain amino acids notably glutamic and aspartic acids, as well as of nitrate, were hard to reconcile with the apparent role of manganese in nitrate assimilation. Reduced decarboxylation of oxaloacetic acid, especially if this were relatively marked compared to effects of manganese on the isocitric dehydrogenase system, would conceivably lead to such results. Comparison of optimal manganese concentrations required for the respective steps is consistent with this idea (45, 53). The operation of mass action laws, as suggested by Hewitt *et al.* (84), might also lead to nitrate accumulation if peptide synthesis were checked by manganese deficiency. Stumpf & Loomis (85) found an enzyme in pumpkin seed that catalyses the production of glutamohydroxamic acid and ammonia from glutamine and hydroxylamine. The system is specifically activated by low concentrations of manganese (2.5×10^{-3} M). Stumpf & Loomis (85) considered their findings in line with those of Hewitt *et al.* (84) and reported the presence of similar systems reacting with asparagine, aspartic acid, and glutamic acid.

Burström (86, 86a) has pursued his study of the role of manganese in wheat roots. Diamyl acetate caused growth abnormalities and inhibited nitrate assimilation. Manganese or iron reversed the inhibition of nitrate assimilation but not the effects on growth. Burström concluded that the effect of manganese was not specific and that nitrate assimilation and growth were distinct effects. He also raised the question whether manganese catalysed nitrate reduction directly, or influenced other reactions that were prerequisites of this aspect of metabolism.

IRON

Iron porphyrin systems.—All the known enzymatic systems depending on iron involve porphyrin molecules. Catalase and peroxidase are widely distributed in the plant kingdom. The general existence of cytochrome-*c* and cytochrome oxidase in plants is, however, less clearly established than for animal tissues. There are nevertheless numerous records of cytochrome containing systems in certain plants, more especially in embryonic or juvenile organs. Thus, Goddard (87) showed that a cytochrome system was the terminal respiratory oxidase in wheat embryo, Maxwell (88) identified cytochrome oxidase in corn embryo and Davison (89) and Waygood (90) detected evanescent cytochrome systems in germinating pea seeds and in wheat, respectively. Bonner (91) identified cytochrome oxidase in the *Avena* coleoptile and concluded that it might be linked to the oxidation of the C_4 acids of the tricarboxylic acid cycle. Polyphenol oxidase was absent. It is not clear, how-

ever, whether oxidation of succinic acid always proceeds via a cytochrome system. Bonner & Wildman (92) found a succinic dehydrogenase in spinach and could detect no cytochrome oxidase but found a polyphenol oxidase system. Rosenberg & Ducet (93), on the other hand, claimed recently to have identified cytochrome oxidase in spinach in addition to the phenol oxidase. Levy & Schade (94) and Schade & Levy (95) obtained evidence (cyanide and light-reversible carbon monoxide inhibition) of an active cytochrome oxidase system in potato tubers which they concluded could adequately cope with the whole of the respiratory oxygen uptake, and cast doubt on the importance of the polyphenol oxidase system. Goddard & Holden (96) confirmed the results of Schade & Levy but did not commit themselves as to whether the cytochrome or the phenol oxidase was the natural terminal oxidase. Cytochrome oxidase may also persist in root tissues, e.g., carrot, according to Marsh & Goddard (97) where oxygen tension is low.

Lundegårdh (98) has obtained further evidence to support his view that the respiratory energy required for salt accumulation is mediated by iron respiratory enzymes. An enzyme thought to contain the iron porphyrin structure was suggested by Tang & Bonner (99) to be the oxidase responsible for auxin destruction. Waring & Werkman (100) concluded that the splitting of formic acid to hydrogen and carbon dioxide by *Aerobacter* spp. was caused by a complex system in which a cytochrome type of enzyme participated. Granick & Gilder (101, 102) and Gilder & Granick (103) have found that specific iron porphyrin compounds are essential for growth and nitrate reduction by *Haemophilus influenzae*. Gilder & Granick (103) also found that the peroxidase activity of iron protoporphyrin leads to mutual destruction of protoporphyrin and a similar interrelationship may occur with other metal porphyrins possessing peroxidase-like activity, e.g., manganese. Sato & Egami (104) concluded that both nitrate reduction and formic dehydrogenase activity in *E. coli* were due to cytochrome-*b* in which iron underwent reversible valency change.

Recent reviews and extensive data on the occurrence and properties of haematin compounds have been published by Scarisbrick (105), Granick & Gilder (106) and Lemberg & Legge (107), the first with especial reference to plants and yeasts. In addition to peroxidase, catalase, cytochrome-*c* and cytochrome oxidase, Scarisbrick (105) presents evidence of the occurrence of at least two other compounds allied to the cytochrome group and having absorption spectra characterised by bands at 5,600 Å (plant cytochrome 560) and at 5,550 Å (plant cytochrome-*f*). The latter has only been identified in leaves so far. It has been found in all leaves examined and appears to be associated with the chloroplasts, where it accounts for a substantial fraction of the haematin present. It is not inhibited by carbon monoxide and has an oxidation-reduction potential slightly above cytochrome-*c*. Its function is not known. Scarisbrick (105) also reviewed the question of the role of the haemoglobin pigment in legume root nodules.

He concluded that the function is probably that of oxygen carrier since, as might be expected, oxygen tension could readily become limiting to the nodule organisms.

Iron status and heavy metal interrelationships.—The role of iron in chlorophyll production and its relation to other metals is of great interest. The presence, let alone the significance, of iron in protoporphyrin 9 identified by Granick (16) as a chlorophyll precursor has not yet been shown.¹ Hill (108) has pointed out that the ferrous and ferric iron porphyrin compounds are both more stable than the magnesium-chlorophyll bond. The replacement, if it occurs, of iron by magnesium in the magnesium protoporphyrin isolated by Granick (15) as a chlorophyll precursor would thus reverse the equilibrium to be anticipated from the relative stability of metal organic complexes determined by Mellor & Maley (109, 110), Maley & Mellor (111), and Irving & Williams (112). According to Granick & Gilder (106), and the general theory of co-ordination complexes, ferrous porphyrin compounds are less stable than ferric. The replacement of iron in a ferrous complex should thus be achieved more readily than in a ferric complex and this may constitute the first logical reason to support the uncritical but commonly held view, justifiably criticised by Arnon (113), that ferrous iron is the "active" form in chlorophyll production, cf., for example, Kliman (114), Liebig (115), Somers & Shive (116), Thorne & Wallace (117).

Hewitt (118, 119, 120) (and unpublished) studied the relation of several heavy metals to the incidence of iron deficiency in sugar beet, tomato, potato, oat, and kale. The susceptibility of these crops to metal-induced iron deficiency varies widely, beet being highly sensitive, and oat and kale relatively insensitive. Tomato and potato are intermediate. The relative ability of different metals to induce symptoms of iron deficiency in beet fell into a fairly reproducible order as follows: Cd^{++} , Cu^{++} , Co^{++} , highly active; Ni^{++} , CrO_4^{--} , Zn^{++} , Cr^{+++} , Mn^{++} , less active in this order. This order bears no relation to the oxidation-reduction potentials of the simple ions and two, namely Cd^{++} and Zn^{++} , do not even undergo valency change. The views of Somers & Shive (116) accepted by many others cannot be reconciled with these results. Little information exists as to the probable type of organic complexes into which these metals may enter in plants. It is well known, however, that the oxidation reduction potentials of complex ions may differ greatly from, and bear no relation to, the potentials of the simple ions, and it is at present impossible to interpret the observed effects in terms of this property. An especially relevant example is found in data for the oxidation-reduction potentials of metal porphyrin complexes (106, 107).

On the other hand, the order of metal-organic complex stability shown by Mellor & Maley (109, 110) Maley & Mellor (111), Irving & Williams (112) and especially of the numerous metal porphyrin complexes given by Granick & Gilder (106) and Lemberg & Legge (107) may be of great importance in

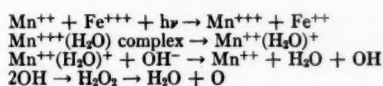
¹ Granick (107a) has suggested that production of chlorophyll or haematin compounds is determined by whether magnesium or iron is inserted into protoporphyrin 9.

the ability of heavy metals to induce iron deficiency. The order of activity found by Hewitt (118, 119, 120) above bears, in fact, a reasonably close resemblance to the order of metal organic complexes, which may be stated approximately as follows: $\text{Cu}^{++} > \text{Ni}^{++} > \text{Co}^{+++} > \text{Fe}^{+++} > \text{Zn}^{++} > \text{Cd}^{++} > \text{Fe}^{++} > \text{Mn}^{++} > \text{Mg}^{++}$, with the important exception of cadmium. Ferric and trivalent manganese porphyrin complexes are comparable in stability to those of copper (106).

The possibility that manganese-induced iron deficiency may arise from competition between manganese and iron in a porphyrin chlorophyll precursor with the production of an inactive manganese containing intermediate was suggested by Sideris & Young (121). It may be noted, however, that divalent manganese lies between ferrous iron and magnesium in the order of probable stability, and the possibility that manganese competes with magnesium rather than iron also merits attention.

Hewitt (118, 119, 120), pointed out that it is not yet justifiable to assume that the various metals able to induce iron deficiency act in an analogous manner, and numerous possibilities require consideration. Sideris (122) found by using radioactive Fe^{59} that iron was associated mainly with the protein constituents of plant tissues, and manganese impeded translocation.

Earlier experiments of Gerretsen on the interrelated hypothetical roles of manganese and iron in photosynthesis were reviewed by Mulder (5). In later work, Gerretsen (123) suggested that the system (shown below) involved a photochemical reduction of ferric iron by divalent manganese as follows:



This scheme is not cyclic, however, unless the final reaction with peroxide produces ferric ions, which is highly speculative.

It may be noted that although Mulder (5) considered that Gerretsen's system (123) confirmed the relationship between iron and manganese inferred by Somers & Shive (116) the direction of valency changes here are the reverse of those originally suggested by Somers & Shive to produce manganese excess effects. Gerretsen (124) subsequently found that manganese slightly stimulated oxygen uptake by crude chloroplast preparations in light at a high potential of 500 mv. He concluded that manganese-induced chlorosis might be due to photo-oxidation of chlorophyll protecting substances.

A possible interrelationship between these metals in which a direct oxidation-reduction reaction does not take place could occur in the tricarboxylic acid cycle. Thus manganese may catalyse oxidation of malic acid by the dehydrogenase systems already reviewed, but malic acid is probably formed from fumaric acid which arises from dehydrogenation of succinic acid. It is likely that in some instances noted the terminal hydrogen acceptor here is a cytochrome system but in other instances ascorbic acid and copper enzymes may be involved. That iron status is related to organic acids is in-

licated by the work of Iljin (125), Hoffer (126) and more recently McGeorge (127) who studied the effect of iron deficiency (resulting from the condition of lime-induced chlorosis) on organic acid status. Chlorotic plants contained more citric and less oxalic or malic acids than green ones. It is not yet clear whether this difference was causal or resultant, but McGeorge (127) found that injection of iron into chlorotic tissues was associated with a reduction in citric acid and an increase in oxalic acid.

An interesting illustration of metal interrelationships with special reference to the function of iron and manganese in porphyrin-protein complexes was observed by Theorell & Swedin (128) and studied further by Swedin & Theorell (129). They found that manganese specifically activated an enzyme termed dihydroxymaleic acid oxidase obtained from *Rumex acetosa*. Cobalt, copper, nickel, and chromium were inactive. Later, Swedin & Theorell (129) concluded that the enzyme was identical with peroxidase, and that whereas peroxidase alone cannot cause oxidation of dihydroxymaleic acid, the reaction proceeds if manganese is also present or if peroxide is added. Manganese produces peroxide during the autoxidation of the substrate which can then cause further oxidation in the presence of the peroxidase. In this reaction they concluded that iron is reversibly reduced to the ferrous state, whereas in peroxidase under normal circumstances (without dihydroxymaleic acid and manganese) the iron remains in the ferric state. They concluded that the single enzyme had distinct properties according to the nature of the substrate and the presence of another activating metal-manganese. Kenten & Mann (130) found that peroxidase can oxidise divalent manganese in the presence of monophenols or resorcinol.

In spite of the suggested ways in which iron and manganese might be interrelated, they nevertheless appear to have some independent roles in plant nutrition. This is shown by the fact that simultaneous deficiency effects of both elements have been recorded and reviewed by Hewitt (118, 119, 120), and by the clear distinction between iron deficiency and manganese toxicity symptoms found in a great many plants studied by Hewitt (131, 132, 133) and confirmed independently by Berger & Gerloff (134) and Morris & Pierre (135). Nicholas (136) analysed a wide range of crops grown in acid and calcareous soils. No relationship existed between the iron/manganese ratio and the incidence of manganese deficiency; and there was no evidence of any iron shortage when excessive concentrations of manganese accumulated in foliage of potato plants grown in acid soils.

BORON

The role of boron in plant metabolism still remains obscure. Rosenberg (137, 138) found that boron or inositol may reverse the growth inhibition by malonate in cultures of *Clostridium saccharobutyricum*. Boron was suggested as a coenzyme in inositol synthesis. Jordan & Anderson (139) concluded that up to 7 p.p.m. boron stimulated nitrogen fixation by *Azotobacter*.

The effects of boron deficiency on the stimulation of tyrosinase activity

observed by MacVicar & Burris (140) [already noted by Mulder (5)] and of increased oxidase activity reported by Reed (141), are difficult to interpret. Addition of boron to chloroplast suspensions reduced oxygen uptake, and a chemical effect rather than one on cell organisation is implied. Possibly the formation of polyhydroxyl-boric acid compounds regulates the rate of cyclic oxidation in phenol oxidase systems. Reed (141) observed a liberation of inorganic phosphate with boron deficiency and this might imply a failure in phosphorylation or in resynthesis of ATP which would occur if for example, the triose phosphate dehydrogenase system were inactivated [cf. Dixon (18, p. 24)]. (Some of the effects should then resemble those due to arsenate poisoning.) Glucose would accumulate due to nonphosphorylation in the absence of ATP. This is one of the effects frequently reported with boron deficiency. The lack of ATP would also presumably impair the synthetic reactions associated with phosphate bond energy and among these are thought to be glutamine and peptide bond syntheses; the other principal effect of boron deficiency, namely, the accumulation of certain soluble nitrogen compounds (possibly glutamic acid?) might thus be explained.

ZINC

The earlier work of Eltinge & Reed (142) and Reed (143) showed that marked cytological abnormalities occurred in tomato in the absence of zinc. Reed (144) used histochemical means to elucidate the effects of zinc deficiency on some enzyme systems in tomato. He concluded that zinc deficiency (like boron deficiency) resulted in the accumulation of inorganic phosphate, and a role in the activation of a phosphate ester enzyme, possibly hexokinase, was suggested. Dehydrogenase activity appeared to be less when zinc was deficient and quinones produced in phenol oxidase systems were not reduced. On the other hand, Tsui (145) found no relation between zinc status and RQ in tomato such as might be expected if zinc activates a phosphate ester system. Chesters & Rolinson (146) also concluded from a study of the effect of zinc on acid production by *A. niger* that no specific effect on carbohydrate metabolism was implied, contrary to the earlier findings of Foster & Waksman (147) that zinc deficiency caused fumaric acid accumulation in *Rhizopus*. Foster & Denison (147a) have now found that zinc is needed for the synthesis of pyruvic decarboxylase by *Rhizopus nigricans* although the metal is apparently not a constituent of the enzyme itself.

Day & Franklyn (148) and Bradfield (149) suggested that zinc plays a role in carbonic anhydrase in plants. The latter showed that such a system exists widely distributed in several plants, and pointed out that many of these were especially susceptible to zinc deficiency. It was present but masked in others, and the need for sulphhydryl groups was inferred. The former found that zinc was associated in a nondialysable form with the protein of the enzyme but a functional relationship was not proved. The enzyme was variously reported as confined to (148) or absent from (149) the chloroplast fraction. Wood & Sibley (149a) found that 15 to 20 per cent of leaf zinc

in oats and 40 per cent in spinach was localised in chloroplasts. Steeman & Kristiansen (150) identified carbonic anhydrase activity in *Elodea* and *Fontinalis* spp. Possibly gaseous exchange of carbon dioxide might be impaired in aquatics. Johnson (151) and Berger & Johnson (74) found that yeast and *Aspergillus parasiticus* tripeptidases were activated by zinc.

Tsui (152, 153) investigated further the effect of zinc deficiency on auxin production and water relations discovered by Skoog (154). Tsui concluded that high osmotic pressures resulted from zinc deficiency due to limited water uptake which in turn was restricted by the failure of the cell walls to grow owing to lack of auxin. Zinc appeared to be needed for a system producing tryptophane which could be converted to auxin without further dependence on zinc status. Nason (155) has found that zinc is also needed for tryptophane formation by *Neurospora*, grown with serine and indole.

COPPER

It is well known that copper activates a group of oxidase enzymes, including tyrosinase or polyphenol oxidase, monophenol oxidase, laccase, and systems oxidising ascorbic acid, all of which may be important terminal oxidases. Before passing to the role of copper itself, the significance of ascorbic acid in oxidation processes in plants, for which good evidence was obtained by James & Cragg (156), should be noted. Davison (89) and Waygood (90) suggested that ascorbic acid undergoes cyclic oxidation and reduction in systems involving coenzyme I, probably a flavoprotein and either malic, succinic, or formic dehydrogenases. The terminal oxidases in pea and wheat were initially cytochrome systems which rapidly disappeared after germination and were replaced by an ascorbic acid oxidase. Catechol oxidase systems were absent.

The importance of copper in tyrosinase (polyphenol oxidase) is not controversial and the properties of this enzyme from mushroom were described in detail by Nelson & Dawson (157). They pointed out that the enzyme has two distinct activities, one the oxidation of tyrosine to dihydroxyphenylalanine (DOPA), the other the oxidation of DOPA and other polyphenols. Ascorbic acid is catalytically oxidised by reduction of DOPA to tyrosine which then competes more successfully for the enzyme than DOPA. Nelson & Dawson (157) concluded that tyrosinase is distinct from laccase or monophenol oxidase. Mallette & Dawson (158) investigated the catecholase and cresolase properties of mushroom tyrosinase. They concluded that the purified enzyme contained 0.25 per cent copper. This corresponded to 4 copper atoms per molecule (mol. wt. 100,000). Catecholase activity was a direct function of copper content and of purification which led to varied cresolase activity. This was ascribed to detachable fragments of the molecule which, when removed, exposed additional sites of catechol oxidation.

Baker & Nelson (159) and Boswell (160) regarded polyphenol oxidase (tyrosinase) as the terminal oxidase responsible for 85 per cent of the oxygen uptake in potato tubers where DOPA and ascorbic acid were the cyclic hydrogen carriers. Amino acids were considered by Boswell (160) to be likely

hydrogen donors via an oxidative deamination mechanism. Recently James, Roberts, Beevers & DeKock (161) have shown that o-quinones can oxidise amino acids and that polyphenol oxidase accelerates the reaction.

There has been some doubt concerning the existence of true ascorbic oxidase and the role of copper. Thus Barron, DeMeio & Klemperer (162) showed that copper alone would catalyse the oxidation of ascorbic acid, whilst cobalt, nickel, manganese, ferrous and ferric iron were inactive. Barron, Barron & Klemperer (163) attributed the protective action of glutathione to the formation of an inactive un-ionised copper complex in a GSH: copper ratio of 2:1. Stotz, Harrer & King (164) and Lampit & Clayson (165) also considered that the role of copper was nonenzymatic and showed that copper protein systems could greatly accelerate the oxidation of ascorbic acid.

On the other hand, Lovett-Janison & Nelson (166) isolated a copper protein from the crook-neck squash plant which showed powerful "ascorbic oxidase" activity. After repeated purification it contained 0.15 per cent copper. It was 500 times more active with ascorbic acid than with catechol and 13,000 times as active as the earlier reported copper-albumen or gelatin preparations. Powers, Lewis & Dawson (167) extended this work and prepared a copper protein containing 0.24 per cent copper with all the properties of a true enzyme and proportionately more active. Sreenivasan & Wandrekar (168) studied the ascorbic oxidase activity of germinating seeds of *Phaseolus mungo*. Seeds soaked for 24 hours had no oxidase but this increased steadily afterwards. A thermostable extract of seeds soaked for 24 hours protected ascorbic acid from oxidation by ionic copper but activated the oxidase preparation. A dialysed extract possessed greater oxidase activity than the same amount of inorganic copper. They suggested that ascorbic oxidase is a mixture of a specific organic fraction and ionic copper. This would be scarcely distinguishable from a dissociable enzyme system. The evidence to date favours the acceptance of a true copper containing ascorbic oxidase in addition to the nonenzymatic activity of copper.

The controversy about the role of copper in laccase seems to have ended (175a). G. Bertrand (169, 170) originally identified manganese as the activating metal in the preparation obtained from *Rhus succadanea*, lucerne, and other plants. Keilin & Mann (171, 172) later showed, apparently conclusively, that laccase was a blue copper-protein containing 0.14–0.24% Cu. It was found to oxidise *o* and *p* diphenols but not monophenols. Recently, D. Bertrand (173, 174) and D. Bertrand, Belval & Legrand (175) have reopened the question. They stated that copper as an impurity was difficult to remove from the laccase preparation obtained from *R. succadanea* but that the purest preparations had a Q_m of 73,000 with *p*-phenylenediamine and contained 0.15 per cent manganese and only 0.03 per cent copper when extraneous copper-contaminated proteins were eliminated by an improved technique. Elimination of copper destroyed the blue colour noted by Keilin & Mann (172) but activity was enhanced. The recent work of Tissières (175a), however, leaves little doubt that laccase is indeed a copper protein.

In spite of some controversial features, it is clear that copper plays a

fundamental role in potential terminal respiratory oxidase systems. For example, phenolases predominate in spinach, potato, and sweet potato according to Bonner & Wildman (92), Boswell (160), and Walter & Nelson (176) respectively. The status of tyrosinase and cytochrome oxidase in potato, however, is now controversial as noted in the discussion on iron. In cereals, ascorbic oxidase predominates according to James & Cragg (156) and Waygood (90).

The localisation of polyphenol oxidase in chloroplasts of spinach beet found by Arnon (177) raises the question whether this enzyme is a true respiratory oxidase in this plant. Arnon (177) speculated whether copper enzymes may participate in the photosynthetic system.

Some years ago Elvehjem (178) found that copper (at 0.1 p.p.m.) was essential for the formation of cytochrome-*a* in yeast, although the *b* and *c* components were scarcely affected. Hill (108) has suggested on the basis of this work that copper may be needed for the formation of the iron porphyrin precursor of chlorophyll and that micro-nutrients may be needed in the production of catalytic molecules each influencing the formation of the preceding one in diminishing concentrations. Arnon's (177) observations may thus have an additional significance. Edmondson & Thimann (179) concluded from a study of deficiency effects and copper chelating substances, that copper was necessary in the biogenesis of anthocyanins by *Spirodella*.

COBALT

No evidence is yet forthcoming that cobalt is an essential element for higher plants although it may greatly influence their metabolism. The bacteria *Lactobacillus lactis* and *Staphylococcus griseus*, however, can, contrary to the earlier reports of Rickes *et al.* (181), utilise cobalt according to Hendlin & Ruger (180) to synthesis either an essential growth factor or cobalt containing vitamin B₁₂. It was found that 1 to 2 p.p.m. cobalt were optimal and 20 to 50 p.p.m. were toxic (180). Hutner *et al.* (182) refer to the need also of *Euglena* and *Chlamydomonas* spp. for B₁₂ and cobalt.

Nickerson (183) and Nickerson & van Rij (184) have shown that cobalt causes mycelial development in yeast whereas other heavy metals do not have this effect. Cysteine reverses the effect, and the early work of Michaelis (185) on the comparative effects of iron and cobalt on cysteine oxidation may be recalled in this context. Cobalt produces a stable cystine-cysteine Co⁺⁺ complex with the intermediate formation of a Co⁺⁺⁺ complex, whereas iron undergoes cyclic oxidation and reduction with the formation of free cystine. The similarity of cobalt and iron with yet an important distinction might also account for the ability of cobalt to induce iron deficiency. Nickerson (183, 184) attributed the action of cobalt on yeast to its ability to form a cysteine complex which would be presumably eliminated from reversible oxidation and reduction systems. The effect of magnesium deficiency in producing mycelial development of *C. welchii* (26) is consistent with these results on sulphhydryl metabolism. Nickerson & Zerahn (186) concluded that yeasts accumulate cobalt in an organic complex or possibly as metaphosphate al-

though no evidence of essentiality for this element was found. The great accumulation of cobalt, nickel, and several micronutrients by microorganisms has also been observed by Mitchell & Tomic (187).

MOLYBDENUM

Deficiency effects and requirements.—Much of the earlier work on the importance of molybdenum to higher plants and on its role in nitrogen metabolism and nitrogen fixation has already been reviewed by Mulder (5).

Hewitt & Jones (188, 189 and unpublished) have continued the survey of molybdenum deficiency in crop plants. Acute deficiency effects have been obtained in lucerne, alsike clover (*T. hybridum*) and red clover all grown with nitrate nitrogen, in several *Brassica* species including rape, marrow-stem kale, hungrygap kale, Brussels sprout, swede, cauliflower, and Savoy cabbage and in lettuce, tomato, and sugar beet. Less acute effects, presumably reflecting either lower requirements or greater seed reserves as suggested by Wilson (190) for beans showing "scald" in acid soils, have been obtained with celery, rye, barley, oat, cocksfoot grass and rye grass, dwarf bean, broad bean, and pea.

The visual symptoms associated with molybdenum deficiency may be summarised as follows. Clovers and lucerne show pale foliage followed by a dull grey-green marginal paling, wilting, incurling and finally leaf withering to a pale buff or white papery scorch. Sugar beet shows paling followed by wilting of the middle and basal parts of the older leaves leading to leaf withering and finally death of the plants. Lettuce seedlings become a pale yellow, older leaves are incurled, scorched at margins and finally withered, followed by death of the plants. Tomato seedlings show upcurling of margins, diffuse intervenal mottling, scorching commencing at the tip leaflet of oldest leaves followed by complete withering of progressively younger leaves until the plants are killed. *Brassica* species differ in certain important respects from the other crops studied. In seedling stages older or middle expanding leaves are affected and usually show pronounced forward cupping, intervenal yellow green or yellow mottling and marginal limpness, wilting and then papery scorching followed by leaf withering, occasionally preceded by wilting of the petioles. If the plants do not have access to any additional molybdenum these symptoms progress until death of the plant occurs. It has been found however, that recovery may readily occur but if it is only temporary, later symptoms develop in a different manner. Middle expanded leaves break down or develop abnormally, leaf margins turn brown and petioles grow without lamina. Death of the growing point may occur. These effects have not been observed in species other than *Brassica*. They account for the appearance of "Whiptail" characteristics of cauliflower and broccoli in acid soils, first produced in sand culture plants by Hewitt & Jones (191) and since frequently confirmed at Long Ashton. Hewitt & Jones (188, 189) suggested that, whereas molybdenum appears to be freely withdrawn from older leaves in most crops, this may not be so after a certain stage in *Brassica*.

The consistent production of molybdenum deficiency symptoms in clover types grown with nitrate nitrogen in the experiments of Hewitt & Jones (188 and unpublished), and the observations of "scald" in beans by Wilson (190) confirm the view that molybdenum is essential for leguminous crops, even when given fixed nitrogen (as nitrate). The response of subterranean clover in acid soils to molybdenum observed by Anderson & Thomas (192) showed that rates of only $\frac{1}{4}$ to 4 oz. per acre were necessary to stimulate symbiotic nitrogen fixation; there was frequently no response at all to molybdenum when fixed nitrogen as nitrate of soda was given, since requirements were even lower but still positive. This view has been confirmed by Anderson in a private communication. On the other hand, both the pot experiments referred to above and the field trials by Mitchell (193), Plant (194, 195), and Waring, Shirlow & Wilson (196) have shown that the molybdenum needs of *Brassica* crops are much greater (4 ozs. to 4 lbs. per acre) and their high requirements are therefore a subject of especial interest.

The role of molybdenum.—The possibility that molybdenum may perform a multiple function in plant nutrition was foreshadowed by Mulder (197) and later by Burström (86), who pointed out that nitrate assimilation may be merely incidental to some more fundamental aspect of the role of molybdenum. There is now evidence to suggest that molybdenum may influence growth apart from its effect on nitrate (or nitrite) reduction. Thus, Vanselow & Datta (198) concluded that molybdenum was needed by *Citrus* plants when given ammonia as a source of nitrogen, thus indicating that molybdenum may still be needed when reduced nitrogen compounds are available instead of nitrate nitrogen. In actual fact, these workers only showed that molybdenum was still needed (perhaps at a lower level) when nitrate and ammonia nitrogen were present together, each in normally adequate amounts. This observation has been completely confirmed at Long Ashton for rape, kale, and tomato where death of the plants occurred in the absence of molybdenum with nitrate only and with nitrate plus adequate nitrogen as ammonia. That ammonia nitrogen is readily utilised for the synthesis of amino acids without molybdenum has been shown by Mulder (197) and by Hewitt, Jones & Williams (unpublished) using a paper chromatography technique applied to extracts of foliage of tomato and cauliflower, whereas with nitrate nitrogen, Hewitt, Jones & Williams (84) found that amino acid production was dependent on the presence of molybdenum. However, the absolute need of molybdenum for normal development when higher plants are supplied with ammonia nitrogen only still remains to be shown. Experimental difficulties with the use of ammonia compounds alone limit progress here at present as was found by Vanselow & Datta (198). It remains to be seen whether the recent improvements in technique for the elimination of molybdenum introduced by Hewitt & Jones (189, 191) and Stout & Meagher (199) can establish a positive molybdenum requirement for *A. niger* under these circumstances, contrary to the findings of Steinberg (200) and Mulder (197).

The condition known as "Whiptail" in cauliflower and broccoli plants and

occasionally found in other *Brassica* types in acid soils [Plant (194, 195) and unpublished] and in culture plants by Hewitt & Jones (188, 189) may, as found by Wilson & Waring (201), be preceded by the mottling typical of molybdenum deficiency in culture plants and associated with high nitrate concentrations. Often, however, "Whiptail"-affected plants have large dark green older leaves indicative of considerable nitrogen assimilation. This condition has now been reproduced by Hewitt & Agarwala (unpublished data) by giving levels of molybdenum intermediate between acute deficiency and normal requirements. In other words, a second role of molybdenum in leaf development in *Brassica* might be inferred as suggested by Hewitt & Jones (189) but the possibility that defective leaf development is also due directly or indirectly to abnormal nitrogen metabolism cannot be discounted.

Millikan (202) found that the injurious effects of several heavy metals, including cobalt, zinc, manganese, and copper on flax in water cultures, and their ability to induce iron deficiency chlorosis were greatly mitigated by additional molybdenum at concentrations between 1 and 10 p.p.m. Hewitt (119, 120, 203) found, however, that with sugar beet in sand culture the opposite occurred. The chlorosis was greatly accentuated by molybdenum at concentrations between 5 and 10 p.p.m. Hewitt (203) extended the work to include the effect of source of nitrogen. It was shown that the molybdenum interaction occurred in the presence of nitrate or urea, often more marked with the latter. These results support the suggestion that molybdenum may affect plant metabolism and especially iron nutrition independently of the source of nitrogen.

During a study of possible systems for nitrate reduction, further evidence of multiple roles of molybdenum has been found recently by Hewitt, Agarwala & Jones (204), in relation to the effect of molybdenum on ascorbic acid status. A significant reduction in ascorbic acid level to 25 to 50 per cent of normal was found in foliage of molybdenum deficient cauliflowers, cabbage, kale, sprout, tomato, beet and other crops. Injection of microgram quantities of molybdenum caused a marked increase in ascorbic acid in 24 hours and the level approached that of controls in 3 to 5 days. Injection of molybdenum into normal plants produced no further increase in ascorbic acid content.

Interpretation of these results is not yet possible. The synthesis of ascorbic acid from a limited precursor, the reduction of dehydroascorbic acid by molybdenum activated dehydrogenase systems possibly similar to those studied by Davison (89), Waygood (90), and James & Cragg (156), or the activation of dehydroascorbic acid reductase studied earlier by Crook & Morgan (205) would be possible explanations. Pinsent (206) has shown that the formic dehydrogenase of *B. coli* is activated by molybdenum in the presence of nitrate or nitrite but not in their absence. The effect of molybdenum deficiency in causing nitrate accumulation might also result in destruction of ascorbic acid in addition to that due to known oxidase systems. The results are, however, contrary to what would be anticipated if molybdenum activated a direct reaction between nitrate (or its derivatives) and ascorbic acid.

It is not clear at present what nitrogen substrate is actually concerned in possible systems activated by molybdenum. Although nitrate accumulates in foliage, nitrite does not under conditions of molybdenum deficiency studied by Hewitt (203) and Wilson & Waring (201), although it does with manganese deficiency under anaerobic conditions according to Jones, Shepardson & Peters (207). On the other hand, Hewitt (203) found that homogenised foliage of plants grown without molybdenum had a greatly lowered ability to reduce added nitrite *in vitro* at 37° C. compared with normal plants. The failure to observe accumulated nitrite might be explained if whatever system were involved could reduce both nitrate and nitrite, but the latter more rapidly (as might be expected). That this is likely appears from unpublished work at Long Ashton with *A. niger* cultures grown with low and normal levels of molybdenum. The concentrations of ascorbic acid found in normal and molybdenum deficient foliage by Hewitt, Agarwala & Jones (204) is consistent with the relative ability of homogenised foliage of plants grown with or without molybdenum to reduce added nitrite and such a reaction might occur *in vivo*.

Molybdenum tolerance.—The remarkable tolerance of plants to molybdenum is a matter of considerable interest. Comparison of the range between deficiency and excess shows that this exceeds by 50 times or more the corresponding range for other micronutrients. Thus, for manganese the respective levels in culture solutions for deficiency and excess effects in cauliflower may be .02 and 20 p.p.m.—a range of 10^3 , whereas for molybdenum the threshold of deficiency occurs between .00005 p.p.m. and .0005 p.p.m.; marked toxicity is only produced above 25 p.p.m. [Agarwala (unpublished)]. This is a range of 5×10^4 . The actual concentration in leaf dry material shows a similar range for these elements. Cauliflowers given 10 p.p.m. molybdenum in culture solution contained up to 2,000 p.p.m. in the leaf dry material (unpublished data) compared with .02 to .1 p.p.m. in deficient material (188).

Warrington (208) and Brenchley (209) gave excess of molybdenum to tomato, other Solanaceae, and flax. Blue or golden pigments due to complex formation with anthocyanins or tannins were observed. Effects resembling ammonia toxicity in cauliflower given excess molybdenum have occasionally been recorded at Long Ashton. This is consistent with the known effect of molybdenum on nitrate reduction and amino acid production shown by Hewitt, Jones & Williams (84). Evidently potentially great activation of molybdenum sensitive systems is not normally injurious.

SULPHUR

Anderson & Spencer (210) reported that sulphur deficiency in subterranean clover prevented effective nitrogen fixation by the root nodule organisms, due to a specific response in the host plant. Thomas, Hendricks & Hill (211) have also found a relationship between sulphur and nitrogen nutrition of lucerne in culture experiments. Plants low in sulphur and grown with high nitrate concentrations lost nitrogen from the total available whilst those rich in sulphur and grown with low nitrate concentrations gained nitrogen in addi-

tion to the total given in solution. It thus appears that sulphur metabolism is closely linked with nitrogen fixation and possibly with the reversal of this process.

GENERAL CONSIDERATIONS

Arnon (private communication) has introduced the concept of the "total metal effect" as a factor of fundamental importance to the role and inter-relationships of mineral elements in plant nutrition. This section is intended to illustrate this concept in terms of the foregoing discussion.

Activation and essentiality.—An interesting example of metal activation relevant to this discussion is the phosphoglucomutase enzyme of muscle. It was found, according to Stickland (212), that two activating metals were required. One was magnesium, the other could be one of several. Only chromium (at greatly reduced efficiency) could replace magnesium and produce single metal activation.

Multiple activation is perhaps most noticeable with manganese and magnesium, and presumably in such circumstances the former does not function through valency change but on account of its similar ionic radius, valency, or complex stability compared with magnesium. Divalency appeared to be the principal requirement for metal activation of yeast carboxylase studied by Green, Herbert & Subrahmanyam (213). Divalent ions of manganese, magnesium, iron, cobalt, cadmium, and zinc were all effective, whereas mono and trivalent ions were not. The theory of a "bridge" between enzyme and substrate due to combination of the metal with both was postulated. The numerous plant, yeast, and animal peptidases described by Berger & Johnson (74, 75, 78), Smith (214 to 217), Smith & Slonim (218), and Smith & Polglase (219) also appear to require some type of co-ordination bridge, e.g., a five-membered chelate ring linking the substrate and metal by the amino groups (219). Here, however, specificity may be greater; in some systems cobalt or manganese are effective whilst magnesium and zinc are not, whereas in others magnesium and manganese are active but cobalt and others are not.

These considerations clearly affect the application of the *Criteria of Essentiality* established by Arnon & Stout (220) to test the essential character of a mineral element. Up to the present time few exceptions have appeared but it is evident that in many instances metals of biological importance can be replaced in individual systems at least *in vitro* by others not yet recognised as essential. So far as known, no single essential element can be completely replaced by another. Experiments to test whether deficiency of an essential element can be rectified by giving several others simultaneously have been few. Such a replacement might occur if the deficient element activated several systems each of which could be activated by an alternative metal, albeit different for each system, but no evidence for this possibility is yet forthcoming.

The demonstration, however, that certain metabolic systems may be activated by apparently non-essential elements such as cobalt or nickel indi-

cates that they may nevertheless influence metabolism in a significant manner. Thus the early idea of "stimulation at low concentrations and toxicity at high ones," practically disclaimed during the period when the true essential nature of the micronutrient elements was being widely established and understood, may in fact be justified with respect to the apparently unessential elements. The stimulatory effects observed by Roach & Barclay (221) when sprays containing nickel and several micro elements were applied to crops may reflect a response of this type.

Total metal effect.—The fact that a metal appears capable of activating more than one enzyme system leads to interesting speculation when there is a partial deficiency or an excess of the metal concerned. The possible significance of this point has already been indicated in relation to effects of manganese concentration on amino acid status and an explanation in terms of the possible differential effect of manganese on the fate of keto acids in the tricarboxylic acid cycle was suggested. Magnesium provides another example where interesting effects might occur. Thus, to obtain full activation of yeast hexokinase about 8×10^{-3} M Mg^{++} concentration is necessary (20) but the activation curve shows no marked optimum. In the isocitric dehydrogenase system (45) maximum activation is obtained with 2.5×10^{-3} M Mg^{++} and there is a sharp optimum, above which activation is reduced. As already noted, manganese activates the enzyme in a similar manner with greater efficiency. It is conceivable, therefore, that different concentrations of magnesium may influence the relative activity of reactions occurring in glycolysis and in the operation of the tricarboxylic acid cycle in a complex fashion. The nature of this interaction would probably be materially affected both by the concentration of available manganese and by the relative competition between the enzymes for limited amounts of magnesium.

The possibility of competition for the total available metal by several enzyme systems was also postulated by Waring & Werkman (100) in a study of the effects of iron deficiency on *Aerobacter* sp. It was found that when iron was deficient the cytochrome oxidase systems of the cell remained relatively well activated whilst other iron-dependent systems were impaired first.

Another aspect of total metal effect concerns the competition between two activating metals for a single enzyme system. An example of this possibility is the citrate oxidation in kidney studied by Hartman & Kalnitsky (222). Manganese was found to be an efficient activator at low concentrations and magnesium produced activation at high concentrations. Intermediate levels of magnesium, however, produced a state of inactivation owing to successful competition with manganese whilst still not being present in concentrations high enough to give appreciable activation. This type of competition does not, however, apply to the isocitric dehydrogenase (45). Unpublished experiments by the reviewer at Long Ashton have shown that partial manganese deficiency in tomato is greatly accentuated by joint traces of cobalt, nickel, chromium, and cadmium. A similar relationship may be involved here, although the work was actually intended to test whether partial replacement or reduction of deficiency effects would occur.

It is not unreasonable to suppose that the interrelationships and balance of the nutrient metals influence the activity *in vivo* of certain systems with far reaching effects on the equilibria attained in others, as may be inferred from the complex interaction between molybdenum, iron, and heavy metals already noted. The effects of the relatively unselective uptake by plants of nonessential but physiologically active metals is also of importance in the assessment of metal status. The facts of enzyme linkage elaborated by Dixon (18) serve to emphasise the complexities to be expected in the elucidation of metal interactions.

Other effects.—It is well known that complex metal organic compounds have oxidation-reduction potentials quite different from those of the simple ions. The formation of a complex between metal and substrate may therefore modify the oxidation-reduction potential of the ligand and thus activate it in the presence of the appropriate enzyme. Justification for this view may be seen from the observation of Hill & Michaelis (223) that the oxidation potential of alloxanthin is materially changed in the presence of iron.

It is convenient to recall here that the reaction attributed to enzymes isolated *in vitro* may not entirely reflect their activity (and therefore of the metals associated with them) when present in the organised cell. The discovery by Keilin & Hartree (224) that the coupling of xanthine oxidase and catalase leads to an entirely new reaction between alcohol and peroxide, not possible with either alone, may anticipate other similar examples.

Arnon (225) has independently expressed fundamental views in almost identical terms and closely coinciding with certain of the ideas discussed in this section. The writer wishes to acknowledge the opportunity given to read the manuscript which was not seen until shortly after this review had gone to press.

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A CRITICAL SURVEY OF THE PHYSICAL BACKGROUND OF PHOTOSYNTHESIS*

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INTRODUCTION

During the last 30 years the methods employed to study photosynthesis have alternated between application of pure organic chemistry and the use of physical and physico-chemical procedures. The former, in the hands of Willstätter *et al.* (1) and later of Fischer (2), gave us a full understanding of the structure of chlorophyll, while the latter, used for quantitative measurement of the over-all gas exchange of photosynthesis, gave us an abundance of information about the influence of external conditions on the photosynthetic rate, quantum yield, etc. Warburg (3), in his pioneer work in this field, introduced the use of manometers. Later, other methods for the measurement of the gas exchange were developed, i.e., calorimetry, electrolytic conductivity measurements, measurements of the oxygen electrode potential, spectroscopic methods, measurements of the heat conductivity of gases, and the use of the quenching effect of oxygen on phosphorescence of dyes. Some of the more important earlier studies, mostly by the manometric method, are: Warburg's (3) early work; van Niel's (4) and Gaffron's (5) studies of purple bacteria and Gaffron's discovery of photoreduction in green plants; Emerson & Arnold's (6) results on photosynthesis in flashing light; Hill's (7) discovery that isolated chloroplasts are able, when illuminated, to reduce substances of high oxidation potential; Kautsky's (8) observation that the chlorophyll fluorescence in plants shows transient changes of its intensity during the induction period and that the intensity of the steady state fluorescence depends upon external conditions.

Because radioactive isotopes (which could be used to follow the chemical course of photosynthesis) have become available, the main progress in the last few years has again been made by the use of organic chemistry. The first studies of Ruben & Kamen (9), who worked with small quantities of the short-lived C^{11} , showed how powerful radioactive isotopes could be used as tools for solving chemical problems of photosynthesis. After long-lived C^{14} became available, Calvin & Benson (10) in California successfully used it to show that phosphoglyceric acid is the first stable intermediate of photosynthesis. The confirmation and implications of these results are discussed in the article by Gaffron & Fager (11) in this volume. However, since the main point of disparity remaining between the results and interpretations of the California group and those of the Chicago group plays a major role in the discussions of this article, it needs to be discussed here.

The fundamental question concerning the photochemical part of photo-

synthesis may be stated thus: "Does the photolysis of water result in the production of a general reducing agent," carbon dioxide reduction being entirely a set of dark reactions using this reducing power? The idea that photosynthesis is actually a reversal of tissue respiration with respiration enzymes acting as a primary substrate of photochemical reduction has a strong appeal to biochemists. Van Niel (4) has interpreted certain results on dark and light metabolism of purple bacteria by this hypothesis, and Wassink (12) believes that some observations on the behavior of chlorophyll fluorescence support it. On the other side, general experience in the field of photochemistry makes this assumption very improbable, as the present writer has often pointed out. According to his judgment, observations on the fluorescence of chlorophyll in photosynthesizing cells speak strongly against it. Benson & Calvin (13) however, interpret some of their results as proof for the correctness of the above-mentioned assumption, while the Chicago workers (11) base their point of view on the fact that their results are somewhat different from those of the California group. They have found no indications of a general reducing power and they regard it as more probable that the 2-carbon carbon dioxide acceptor may be reduced photochemically and then act as a specific reducing agent for carbon dioxide by combining with it in a carboxylation reaction which results in the formation of phosphoglyceric acid. A part of the following discussion is a re-evaluation of interpretations presented at a time when the role of phosphoglyceric acid in photosynthesis was unknown (and is, therefore, based only on physical and physical-chemical observations). The same compound must be considered in some questions of chemical kinetics and especially in the problem of induction periods which we will have to discuss. Another problem belonging in our review is the role of chlorophyll in plants and *in vitro* for sensitizing photochemical reactions. While this problem is by no means solved, indications exist as to how the solution can be approached, and studies of the chlorophyll fluorescence in plants and in organic solvents will undoubtedly play a role in this connection. The selection of the topics covered cannot help but be somewhat arbitrary. The reviewer had to rely on his own judgment in choosing those observations which he regards as basic. In the same way the interpretation of the results is, by necessity, colored by personal views. A description of all of the items which might fall under the heading of this article and of all interpretations which have been proposed would be too long to be printed and too tedious to be read.

CHLOROPHYLL FLUORESCENCE AND PHOTOCHEMISTRY IN PHOTOSYNTHESIZING CELLS AND *In Vitro*

It was mentioned in the introduction that the fluorescence yield of chlorophyll is often influenced by external or internal factors which influence the photosynthetic rate. A rise of the fluorescence yield in green plants and purple bacteria is connected with a lowering of the photosynthetic rate and often the two show a perfect anti-parallelism of their time courses. Under certain

conditions, the photosynthetic rate may change without corresponding changes of fluorescence yield; under others, the time courses of the changes of the two are out of phase or may even temporarily run parallel instead of anti-parallel. The matter becomes still more complicated since transient changes in the photosynthetic rate during the induction period can be quite different for the two processes of carbon dioxide uptake and oxygen production. It is evident that not only a great many observations on plants and other photosensitizing cells must be made before a theoretical picture in accord with all observations can emerge, but that exhaustive studies of the relation between chlorophyll fluorescence and photochemistry *in vitro* are also needed. The four groups who studied the chlorophyll fluorescence of photosynthesizing cells used in principle the same observational method, but differed somewhat in the type of experiments they performed. Kautsky *et al.* (8) were the first to do systematic work in this field. Franck *et al.* (14 to 17) used instruments able to measure very rapid changes of yield and to register the yield at the very start of the irradiation period. McAlister & Myers (18) were the first to measure the time courses of fluorescence and of photosynthetic rates simultaneously. Wassink and his school (12) did the most extensive work and studied, in addition to green algae, the chlorophyll fluorescence in purple bacteria and in diatoms. Besides Kautsky's (19) early attempt at a theoretical interpretation (which does not seem to have stood the test of time), only two theoretical pictures have been proposed which claim to account for all observations; the one by Wassink (12) and the other by Franck *et al.* (14 to 17). Wassink's theory is based on the assumption that any influence that produces a rise of fluorescence intensity must be an influence which hinders transfer of energy from the excited chlorophyll to the substances which use it for photosynthetic purposes, thereby reducing the utilization of the light for photosynthesis. In other words, he assumes that the same kind of antagonism between light emission by fluorescence and the use of the excitation energy for photochemical purposes exists in polyatomic dye molecules, as was found about 30 years ago for monatomic and diatomic molecules. However, there exists a great difference in the relation between fluorescence and photochemistry in these two types of molecules. In the simple ones, the quantum yield of fluorescence goes down from the value one to zero, with rising concentration of substances able to use the light energy photochemically, while the chlorophyll fluorescence yield in the cells varies only between the extremes of .001 to .005, even when photosynthesis varies between zero and its maximum value. Franck, therefore, pointed out that in polyatomic molecules like chlorophyll quite other processes than the occurrence of photochemical reactions are responsible for quenching of the fluorescence. Studies of chlorophyll and other dyestuffs *in vitro* (to be discussed below) showed that contact of dyes with photosensitive substances is not necessarily connected with a lowering of the fluorescence yield. It may in some cases even cause a rise of the fluorescence yield and in others leave it practically unchanged. Since, for the time being,

it cannot be predicted which of these possibilities will occur in the cells, it is left to empirical tests to find out. According to Franck and Shiau (17), the observations can easily be interpreted by the assumption that in green plants and bacteria the fluorescence yield is very little dependent upon the photosensitivity of material in contact with chlorophyll; however, if certain surface-active substances, called "narcotics" by Warburg (3), are brought in contact with chlorophyll the fluorescence yield rises by a factor of 3 to 4 (20). Similar increases in the fluorescence yield in plants and purple bacteria always appear when photooxidation or oxidations by photoperoxides occur in the cells. This similarity is the reason why the reviewer introduced the assumption that photooxidation of easily oxidized substances produces a "natural narcotic" in plant cells and purple bacteria. Evidence has been presented which indicates that the formation of such a "narcotic layer" has the important function of checking damaging reactions like photooxidation and of controlling photosynthetic activity in higher plants according to the needs of the plant (16). Wassink, however, rejects the idea of a natural narcotic because it does not appeal to him. He prefers to introduce special assumptions for each individual case. It is impossible in this survey to discuss the great abundance of observations on the fluorescence, and there is also no need to do so because several long survey articles on this subject exist and another written by Wassink (which he kindly showed to the present writer in manuscript) is soon to appear in *Advances in Enzymology* (12). We will confine ourselves to the discussion of a few pertinent facts and a short interpretation (not published elsewhere) of Wassink & Kersten's (21) observations of fluorescence of diatoms. However, since all of these interpretations are intimately connected with results of studies of the behavior of fluorescent polyatomic molecules *in vitro*, which are mostly to be found in the literature not read by biologists, it seems best to present first a brief description of those results with chlorophyll *in vitro* which are directly related to our problem.

INTERNAL CONVERSION OF EXCITATION ENERGY IN COMPLEX MOLECULES

In accord with theoretical expectations, dyestuff molecules dissolved in a photochemically-stable solvent are able to fluoresce. However, a special explanation is necessary for the observation that in most of the dyes the fluorescence yield is smaller than one and in many cases it is negligibly small even if photochemically unstable impurities are carefully avoided. Theoretical and experimental research revealed that the fluorescence yield is lowered by a process called internal conversion which occurs only in complex molecules (22). This consists in a conversion of excitation energy of the electronic system of the complex molecule into oscillation energy of the atomic constituents of the molecule. Thus the light energy absorbed by the molecule is transformed into intramolecular heat movement, which, in a condensed system, is quickly distributed intermolecularly into general heat movement

of the molecules in contact with the one which originally became excited. For reasons not entirely understood, conversion usually proceeds in such a way that radiationless transitions occur to the next lower excitation level. Thus if chlorophyll is excited with blue light which raises the electronic state of the molecule to the second excited singlet state, the molecule undergoes internal conversion to the first excited singlet state. Indeed, this radiationless transition is so rapid that no fluorescence is observed which corresponds to radiative transitions from the second state to lower states and the energy difference between the second and the first excited states is dissipated as heat. The first excited state, thus populated indirectly, lives just as long as when it is excited by red light and, correspondingly, shows its typical red fluorescence emission. However, an internal conversion to the next lower state also occurs with the result that the fluorescence yield becomes less than 1. In the case of chlorophyll, the highest fluorescence quantum yield observed *in vitro* is approximately 1/10, while it is about one hundred times smaller in cells.

ROLE OF THE METASTABLE STATE OF CHLOROPHYLL FOR FLUORESCENCE AND PHOTOCHEMISTRY

The next lower electronic state of the chlorophyll is, in analogy to other dyestuffs of similar type, supposed to be a so-called triplet state. Lewis and co-workers [cf. review by Kasha (23)] have shown that this long-lived state is responsible for the occurrence of dye phosphorescence. It is called a metastable state on account of its very small transition probability for reverting to the ground state by radiation. Therefore, its emission spectrum is so weak that it is difficult to observe it. In fact, even the reported direct observations of the expected phosphorescence are doubtful. Kautsky *et al.* (24) observed an after-glow at room temperature, which could not be confirmed by Linschitz (25), and the result of Calvin & Dorough (26), who believed to have found a phosphorescence at very low temperature, is in contradiction to Livingston's (27) observations. Livingston's method was sensitive enough to observe very weak emission provided that the light emission contained wavelengths shorter than 9000 Å. Because he observed no phosphorescence, he assumes that, if observable, it may be found in the region of still longer wavelengths. The results of Kautsky *et al.* (24) and Calvin & Dorough (26) may be explained by the presence of impurities. If the chlorophyll phosphorescence has indeed a spectrum lying beyond 9000 Å, the molecules excited to the lowest triplet level would contain an energy smaller than 32 kcal.

In spite of the uncertainty of the exact position of the metastable state in the energy scale, there is ample evidence that (a) internal conversion rather than the presence of photosensitive substances is responsible for the limitation of the fluorescence yield of chlorophyll dissolved in certain organic solvents; (b) that as a result of internal conversion there is a transition of chlorophyll into a metastable (triplet) state which lives much longer than

the primarily excited singlet state; and (c) that it is predominantly the energy stored in this triplet state which is used for the photochemical reaction, while the percentage contribution of the originally excited singlet state is negligibly small. Evidence in favor of point (a) is presented in general discussions of internal conversion [Franck & Livingston (22); Franck & Sponer (28)]. One experiment may be discussed as an example. Chlorophyll *in vitro* is known to be a very efficient sensitizer of oxidation processes. The first reaction of this type quantitatively studied was the chlorophyll-sensitized oxidation of allylthiourea in acetone by Gaffron in 1927 (29). The reaction proceeds with a quantum yield so near to one that Warburg & Burk (30) use it as a chemical actinometer for measurements of photochemical quantum yield. Allylthiourea has no quenching influence on the chlorophyll fluorescence [Livingston & Ke (31)], and only very small concentrations of oxygen, which has a quenching influence, are needed to let the oxidation reaction proceed with a quantum yield of one. No difference in the chlorophyll fluorescence yield is observed between a solution in pure acetone and one in acetone containing allylthiourea and oxygen in sufficient concentrations to permit a high photochemical quantum yield. If the singlet state were responsible for the reaction, its fluorescence emission should be entirely quenched before a photochemical yield of about 1 could be reached. Of course, the light re-emitted by fluorescence is lost for photochemical purposes but in this case it is a loss of only a few per cent of the energy absorbed; i.e., smaller than the accuracy of the efficiency measurements. Such an experiment at the same time proves points (b) and (c). The energy needed for the sensitized reaction must still have been present in the chlorophyll molecule after the internal process which limits the fluorescence emission took place. Otherwise a photochemical quantum yield of 1 is impossible. If the internal transition were to consist in a transformation of the total excitation energy into internal heat movement, it would result in a dissipation of the energy before the thiourea or oxygen could take over the energy by impacts and use it for chemical purposes.

Internal conversion, therefore, must proceed in a way which permits storing a sufficient part of the energy in the chlorophyll molecule itself. There are only two ways of storing the energy, either as Franck & Livingston (22) first believed, by the formation of an energy-rich tautomer of the chlorophyll molecule or by storage in an excited electronic state lying lower than the lowest singlet state. Lewis and co-workers have proved beyond any doubt that dyestuffs have triplet states of suitable energetic position, and Kasha (23) presented evidence that they indeed can be populated from the next higher singlet state by the process of internal conversion. There is no reason to expect that chlorophyll differs in this respect from other dyestuffs. Moreover, the chemical kinetics of the oxidation reactions sensitized by chlorophyll demands the presence of a long-lived excited state. This was pointed out by Gaffron (29) in his early observations.

REVERSIBLE BLEACHING OF CHLOROPHYLL

A phenomenon which is certainly connected with the occurrence of the metastable state and possibly might be the direct observation of it is the process of "reversible bleaching" of chlorophyll in organic solvents first observed by Rabinowitch & Weiss (32). It consists in a small fading of the green color of chlorophyll in strong light. In the dark the chlorophyll color is quickly restored to its original intensity. This process has been carefully studied in recent years by Livingston (27) and by Krasnovskii and co-workers (33), and others. Livingston's latest, still unpublished work (personal communication) permits an estimate of the lifetime of the bleached state. Its value is about 1/1000th second. Such a lifetime would be reasonable if one regards it as the lifetime of the metastable state of the chlorophyll but it can just as well be that the bleached chlorophyll is a short-lived reduced state of chlorophyll, as Franck & Livingston (22) originally suggested. Krasnovskii and his associates (33) also make the latter assumption and base it on photochemical observations. They had indications that substances like ascorbic acid, phenylhydrazine, and dihydroxymaleic acid can reduce chlorophyll in the light while the reaction is reversed in the dark. Another indication that reversible bleaching is connected with the metastable state is the fact that the bleaching is suppressed by the presence of oxygen. It is known from experiments on other dyes that impacts of oxygen always destroy triplet states by enforcing transitions to the ground singlet state by magnetic interaction. These observations prove that, in the course of photochemical reactions, chlorophyll undergoes transient changes in its structure. As do most students of photochemistry, the reviewer is inclined to assume that not only transient changes of the electronic structure but direct chemical participation of the chlorophyll occurs in the course of oxidation-reduction reactions which it sensitizes.

CONDITIONS WHICH INFLUENCE CHLOROPHYLL
FLUORESCENCE *In Vitro*

It was stated above that a general prediction cannot be made as to how the contact of excited chlorophyll with photosensitive substances will influence the fluorescence. A rise or a fall of the fluorescence yield can result, depending on the special structure of the substances in question. Indeed, such behavior is theoretically expected and is experimentally observed in experiments with chlorophyll *in vitro*. We have discussed how, in the absence of photosensitive substances, limitations of the fluorescence yield are imposed by the competition of the process of internal conversion with light emission. The faster the former occurs, the shorter will be the lifetime of the excited state and the lower will be the light emission. Every small change in the structure of the excited molecule and even changes of the surrounding molecules in contact with it will alter the probability of internal conversion

and thereby influence the fluorescence intensity. The reason is a simple one. The radiationless transition of internal conversion can occur only when, by internal atomic motion in the excited molecule, a critical constellation of all the atomic constituents and of their momenta is reached. The time needed to reach the critical constellation obviously depends on the structure.

The same principle holds for all other radiationless transitions; for instance, those radiationless transitions of the electronic system which we call chemical reactions. In case of contact by adsorption or impact between the excited molecule and another molecule, we have to treat both molecules together for the duration of the contact as one correspondingly more complicated molecule. The internal motion of their atomic constituents is coupled to some degree and becomes, therefore, more complicated. In general, the stronger the adsorption the stronger will be the coupling. Thus the time needed to reach the critical point may often be prolonged by contact with other molecules. If the molecule in contact with the excited one is photosensitive, i.e., if it can undergo chemical changes by utilization of the excitation energy, then its contact adds another possibility for a radiationless transition, namely, the chemical one. The question of whether contact with the substance quenches fluorescence or enlarges its yield depends upon whether a postponement of internal conversion or the addition of a new possibility for a radiationless transition has a greater influence. Another complication which has to be taken into account is the possibility that by contact with or by adsorption to another molecule the spectrum of the dye may be influenced. The influence observed in the case of chlorophyll consists in some cases in a shift of wavelengths and in changes of the absorption coefficient. However, in other cases no spectral changes are found. The experiments with chlorophyll *in vitro* to which we refer were mostly carried out by Livingston and co-workers (27, 34) and by a Russian group in Terenin's (33) laboratory.

A few examples may be mentioned: Livingston (27) found that chlorophyll dissolved in very pure hydrocarbons like benzene or *n*-heptane has a very low fluorescence yield, but addition of a very small quantity of water or of methanol lets the fluorescence yield rise to the height of about 1/10 that which chlorophyll usually has in polar organic solvents. Livingston concludes that this fluorescence activating action is caused by the formation of a solvated chlorophyll. The absorption spectrum of chlorophyll is, in pure hydrocarbon, considerably weaker than after addition of the activator and also the position of the absorption maxima is shifted. Krasnovskii and co-workers (33) have shown that the central magnesium atom of the chlorophyll is the spot where the activating action takes place. It is probably the result of coordinate bond formation between the activator and the magnesium. Pheophytin (i.e., chlorophyll after removal of magnesium) does not change its fluorescence intensity and its absorption spectrum by addition of the activators. Addition to a solution of chlorophyll in dry *n*-heptane of phenylhydrazine (a photosensitive substance which is easily oxidized in

a photochemical reaction sensitized by chlorophyll) activates the fluorescence of chlorophyll as long as the concentrations are small. But if it is added in high concentrations it causes quenching of the activated fluorescence. Urethane, a powerful narcotic inhibitor of photosynthesis, which enhances the fluorescence of chlorophyll in plants, has no influence on the fluorescence yield if the chlorophyll is dissolved in ether, acetone, or methanol. These examples may suffice to show how complicated the influences are which substances exert by their contact with chlorophyll. The simple assumption that photosensitization and fluorescence are complementary to each other is neither true for chlorophyll *in vitro* nor *in vivo*.

ENERGY MIGRATION IN CHLOROPHYLL *In Vitro*

All the experiments discussed in the previous paragraph were carried out in very dilute chlorophyll solutions (concentration 10^{-6} to 10^{-8} M), while in the chloroplast the concentration is of the order of 10^{-2} M. It is, therefore, of importance to mention the phenomenon of energy migration from a primary excited dyestuff molecule to other dye molecules, which becomes significant at high dyestuff concentrations. The literature on this subject is very extensive. The simplest case of migration of excitation energy are the ones in which the energy transfer occurs between molecules which are exactly alike. But even under this condition two quite different possibilities occur. The first one, called exciton migration by Frenkel (35) relates to an exceedingly quick transfer of energy between the molecules; the energy remains in each individual molecule for a time which is so short compared to the atomic oscillation periods that the atomic constituents are practically fixed in their positions during the short time of the visit of excitation energy in an individual molecule. This process demands a very strong coupling of the molecules which apparently is not even achieved in a crystal of these organic molecules, as the absence of spectral changes typical for a very narrow coupling indicates (36).

The second possibility for energy transfer is a slow migration in which the energy transfer from molecule to molecule takes a longer time than the atomic oscillation periods. In that case the coupling is too weak to produce a typical change in the absorption spectrum. Förster (37) calculated from the overlapping of absorption and fluorescence spectrum of chlorophyll that at a concentration of 10^{-2} M the energy might be transferred from one molecule to the next one in an average time of about 10^{-11} sec. The same calculation made by Förster for the slow migration of excitation energy between chemically identical molecules can be used for calculation of energy transfer between molecules which are not equal. Such a transfer is called a sensitized fluorescence and was first observed as energy transfer between different species of atoms [(38) cf. (39).] To calculate the probability of sensitizing the fluorescence of a molecule X by light absorbed by molecule Y, one has to take into account the overlapping of the fluorescence spectrum of Y and the absorption spectrum of X. The overlapping of both of these

spectra is usually quite strong if the absorption spectrum of X has a little longer wavelength than the absorption of Y. Under these conditions, the probability of energy transfer from Y to X is indeed greater than the transfer probability from Y molecules to other Y molecules or the transfer between molecules. Several examples of sensitized fluorescence in dyestuff mixtures are known.

Of interest in connection with photosynthesis are studies of this process in mixtures of chlorophyll *a* and *b* dissolved in acetone by Livingston *et al.* (40). The lowest excited singlet state of chlorophyll *b* lies slightly above the first excited singlet state of chlorophyll *a*, and correspondingly the fluorescence emission spectra of chlorophyll *b* overlaps strongly the first absorption band of chlorophyll *a*. Consequently, sensitized fluorescence by light absorbed by chlorophyll *b* is already in evidence at the relatively small concentrations of chlorophyll *a* + *b* of 10^{-4} to 10^{-3} *M*. Because the concentration of chlorophyll is much higher in the chloroplasts, it is obvious that energy absorbed by chlorophyll *b* must be transferred to *a*, and it is most probable that energy absorbed by carotenoids will be efficiently transferred to the chlorophyll by the same mechanism. The corresponding sensitized fluorescence in chloroplasts was observed first by Dutton & Manning (41) and later by Wassink (12). The question arises as to whether by the process of sensitized fluorescence, energy from an excited singlet state of chlorophyll can be transferred to another chlorophyll molecule in such a way that the metastable state is excited in the latter. At the high concentration of 10^{-2} *M*, the process will certainly occur but the transfer probability is much smaller in this case (even if the position of the fluorescence emission spectrum of the singlet-singlet transition might be favorable in respect to the still unknown absorption spectrum of the singlet-triplet transition) because the so-called intercombination transitions, i.e., transitions between electronic states of different multiplicity, are always infrequent. The process of internal conversion is apparently much more efficient for the production of metastable excited chlorophyll molecules.

SELF-QUENCHING OF CHLOROPHYLL FLUORESCENCE *In Vitro*

If the concentration of dye in a solution is raised to values higher than a few times 10^{-3} *M*, the fluorescence yield usually begins to fall. This process is called self-quenching. It is not fully understood, and it is not excluded that it might have a connection with the formation of metastable molecules by the process of sensitized fluorescence (20). In whatever manner chlorophyll molecules in the metastable state may be formed, there remains no doubt that the process occurs and that the transitions to the triplet state are so frequent that the quantum yield of their formation is practically equal to one. For chlorophyll in plants this condition is more rigidly fulfilled than *in vitro* as may be deduced from the fact that the fluorescence yield is smaller in plants than *in vitro*. In other words, all the light quanta absorbed by chlorophyll *a* and *b* will produce indirectly one metastable excited

chlorophyll molecule per quantum. It is probable that preponderantly one kind of metastable chlorophyll molecule will be formed, either chlorophyll *a* or *b*, whichever has the lower lying metastable state. As long as the absorption and fluorescence spectra of the first singlet-triplet transitions are not known, it is unsafe to predict how often the energy of metastable molecules will be transferred from one dye molecule to another. For several reasons the transfer time will certainly be much longer than the time calculated by Förster (37) for the transfer of the excitation of the singlet state. Still, the metastable state may migrate through many molecules if the conditions permit the metastable state to outlive its natural lifetime; i.e., if its lifetime is not very much shortened by internal conversion or by impacts with molecules which destroy the metastable state. If special precautions are not taken, the latter process will be the rule.

NARCOTIC INFLUENCES IN PHOTOSYNTHESIZING CELLS

The discussions in the preceding paragraphs have shown that the fluorescence of chlorophyll in organic solutions can be enhanced by activator molecules. These activator molecules combine with the magnesium of the chlorophyll, and, if every chlorophyll molecule is connected with an activator, the fluorescence yield may rise by a factor of the order of magnitude of 10. The activating molecules belong not only to the class of photochemically inert substances but some are photosensitive; i.e., they are able to make use of the excitation energy of the chlorophyll for photochemical purposes. These results strongly support the assumption that the limitation of fluorescence yield is due much more to internal conversion than to quenching by contact with photosensitive substances, a conclusion which was reached before activators of chlorophyll fluorescence *in vitro* were observed. In green plants and in purple bacteria, it happens that narcotic-acting substances; i.e., surface-active substances, which inhibit photosynthesis at low as well as at high light intensities, are activators of the fluorescence. They raise the fluorescence yield by a factor of about 5 if they are present in concentrations sufficient to suppress totally photosynthetic activity. This factor is comparable to the rises which activators produce *in vitro* and justifies the conclusion that all chlorophyll molecules in the cells are accessible to the narcotic. This statement is not meant to exclude the possibility that, due to morphological factors in chloroplasts, some parts of the chlorophyll may be more accessible than others, but it is one of the reasons which make it unlikely that only one in several thousands of chlorophyll molecules is in such a position that it can come in contact either with inhibitors or with substances (see *Photosynthetic unit*) to be photosynthetically reduced. The fact that a photosensitive substance like quinone, which quenches fluorescence of chlorophyll *in vitro*, lowers the fluorescence yield in plants below the usual value during photosynthesis is regarded as another example that direct contact of chlorophyll occurs with photosensitive and photo-insensitive substances, and that they influence the fluorescence yield in plants in a

manner analogous to their action *in vitro* [Shiau & Franck (17)]. The question arises as to whether in photosynthesizing cells only photo-insensitive substances like narcotics are activators. Observations made by Wassink & Kersten (21) on the relation between fluorescence and photosynthesis in di-

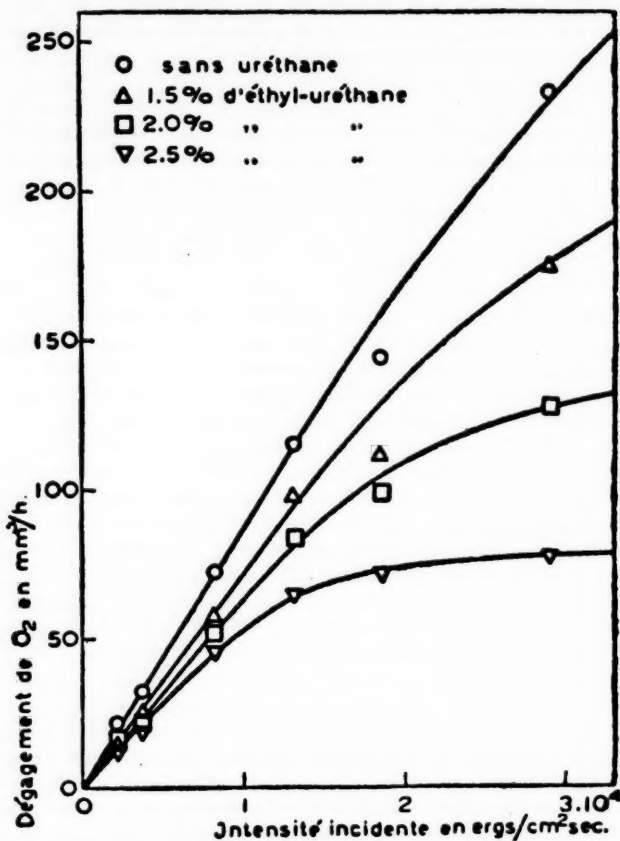


FIG. 1. Wassink and Kersten. Influence of ethylurethane on photosynthetic rates of diatoms (25° C.).

atoms indicate, according to the reviewer's interpretation, that in these cells the photosensitive substances are more effective than narcotics as activators of fluorescence.

Figure 1, taken from Wassink & Kersten's paper (21), shows that the influence of ethylurethane on the photosynthetic rate in diatoms is not the

same as in *Chlorella* and other green algae. In the latter, ethylurethane and other narcotics influence by the same factor the saturation rate and the rates where light intensity is limiting. Indeed, this criterion is usually regarded as typical for a narcotic-acting substance, and it is interpreted as

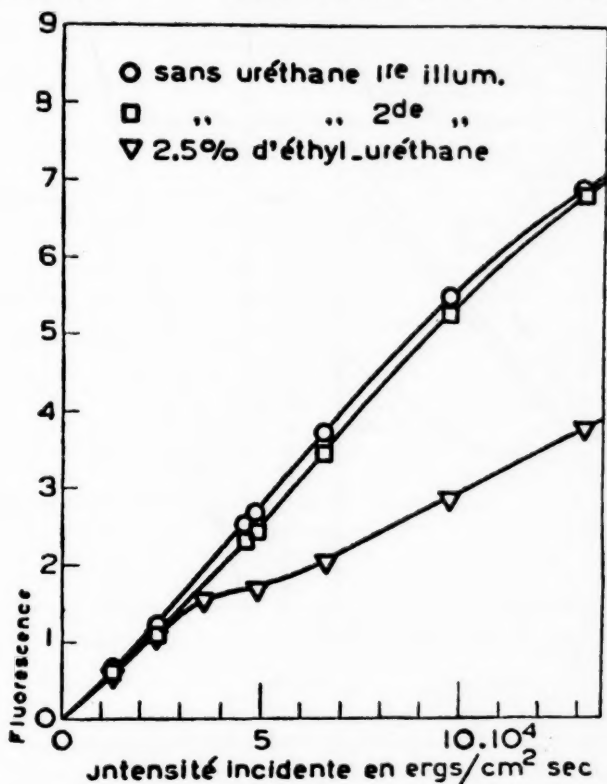


FIG. 2. Wassink and Kersten. Influence of ethylurethane on fluorescence intensity of diatoms (25° C.).

proof that the narcotic is an unspecific surface cover. It covers and thereby inactivates the surface of the chlorophyll-protein complexes to the same degree as the enzymes which are responsible for the occurrence of saturation.

Later in this section we will give a more detailed discussion of this problem. Here it is sufficient to point out that in diatoms the influence of the narcotics is much greater on saturation rates than on rates in the region of low light intensities. The observed curve resembles those which are typical

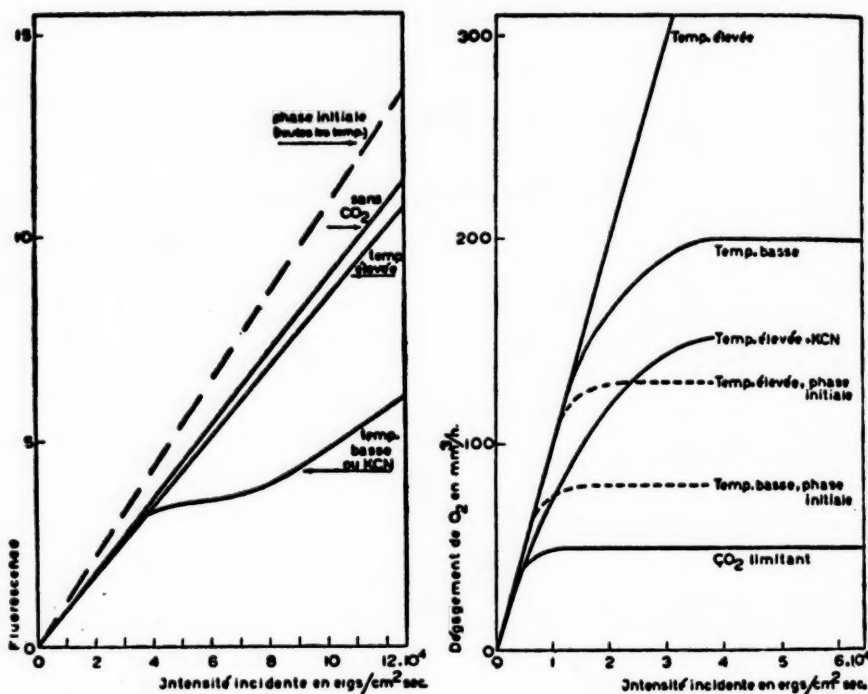


FIG. 3

Wassink and Kersten. Fluorescence intensity of diatoms as function of light intensity under different conditions. Solid lines represent the steady states of fluorescence. Dotted lines represent fluorescence intensities at the start of irradiation periods.

Photosynthetic rates of diatoms as function of light intensity under different conditions. Solid lines represent final values of rates. Dotted lines represent rates at the start of irradiation periods.

for a poison like cyanide. Another difference between the action of ethylurethane in green algae and diatoms is that in the latter higher concentrations are needed to give the same inhibitions. These differences indicate that in diatoms the molecules which are reduced in photosynthesis are adsorbed more strongly by the chlorophyll than in green algae and are, therefore, displaced by the narcotic only if the narcotic concentration is high or if their own concentration becomes low, a condition evidently fulfilled at light saturation. It is a general rule that the fluorescence in dyes becomes stronger by all factors which hamper the internal movements of the atomic constituents of the complex dye molecule; adsorption is such a factor (22). We may, therefore, expect that the fluorescence yield at low light intensities is greater in diatoms than in green algae. Wassink & Kersten (21) found, indeed, that the fluorescence yield is about three times higher in the diatoms. Figure 2 shows the influence of ethylurethane on the fluorescence. Its influence on diatoms is, as the effect on photosynthetic rates indicated, entirely different from that in green algae. In the latter, the addition of narcotics causes the same percentage rise of the fluorescence yield at all light intensities. If the inhibition is strong, the slope of the entirely linear curve is several times steeper than the slope of the fluorescence curve in absence of the narcotic. In diatoms, on the other hand, the influence of ethylurethane at low light intensities is negligible, while at saturation the fluorescence yield drops considerably and then rises slowly again in the region of still higher light intensities. Therefore, we come to the conclusion, already mentioned, that in diatoms the substances engaged in photosynthetic reduction are stronger activators of fluorescence than urethane, which at saturation intensities displaces these substances. It might be emphasized that at low intensities photosynthesis proceeds without any limitations beyond the ones imposed by the light intensity. That follows from Wassink & Kersten's (21) result that the quantum yield measured at low intensities lies between $1/10$ and $1/13$. These are the values identical with the ones found by every observer of green plants [including Warburg & Burk (30)], if the plants are kept in Warburg's buffer 9 as was done here with the diatoms.

The same explanation holds true for either the saturation or fluorescence curves of Fig. 3 [Wassink & Kersten (21)], in which either low temperature or cyanide is responsible for saturation. The only difference between these observations and the ones made in the presence of urethane is that in the latter case at saturation ethylurethane covers the chlorophyll surface, while in presence of cyanide or at low temperature the natural narcotic made by photooxidation will be the surface cover. The formation of the natural narcotic sets in as soon as the chlorophyll surface becomes free as a consequence of the atmosphere containing 20 per cent of oxygen.

Another interesting observation depicted in Fig. 3 is that at the start of irradiation with intensities beyond saturation, the fluorescence yield is as high as at low intensities and drops down to its final value in 10 to 20 sec. The saturation rates, on the other hand, start low and climb in about the

same time to their higher final value. To show that this behavior is in accord with our hypothesis, we need a more detailed discussion of the problem as to why and how the chlorophyll surface becomes empty when saturation is reached at low temperatures or in presence of cyanide, or if carbon dioxide is limiting. The assumption that at saturation, in presence of cyanide, or at low temperatures the photosynthetic apparatus would become empty was an obvious consequence of the hypothesis that carbon dioxide itself or a substance which has added the carbon dioxide is involved in all of the reduction steps which take place in direct contact with the chlorophyll. This hypothesis was used in the survey of the fluorescence phenomena about which the present author wrote in a chapter of "Photosynthesis in Plants" (42). We know now that we have to modify this hypothesis to some degree after Calvin & Benson's (10) work. Let us first discuss the emptying process if saturation is caused by the slowness of carbon dioxide uptake on the premise that Calvin & Benson's assumption of a general reducing agent is right. The only photochemical reactions are then the formation of that reducing agent XH_2 out of X and H_2O . If the consumption of XH_2 in the dark reactions by which CO_2 is reduced is the limiting factor, then a surplus of XH_2 must accumulate until the concentration of X becomes so low that the photochemical production of XH_2 equals its consumption. The chlorophyll surface apparatus will thus be partially deprived of contact with the photosensitive X . A surplus of XH_2 must develop even if this substance is oxidized again by respiration because this process is slower than the photochemical production.

Our concept differs from Calvin & Benson's (cf. Fager & Gaffron's review in this issue). It demands two sets of photochemical reactions: (a) the formation of the reduced C_2 compound written in symbols $R + H_2O + h\nu \rightarrow RH_2 + 1/2 O_2$ followed by the dark reaction $RH_2 + CO_2 \rightarrow \text{phosphoglyceric acid}$, and (b) $\text{phosphoglyceric acid} + H_2O + h\nu \rightarrow \text{triose} + 1/2 O_2$. On these premises the emptying of the chlorophyll surface at saturation if the carbon dioxide uptake in the dark reaction is limiting is a little bit more involved. If we suddenly raise the light intensity from a value just sufficient to cause saturation to a higher intensity, the concentration of the substances to be reduced (R) and phosphoglyceric acid will, at the start of the stronger irradiation be as high as it was at the lower intensity. The RH_2 production out of R and the reduction rate of PGA to triose must therefore rise for a short time to a value surpassing the saturation rate. However, the new production of phosphoglyceric acid by reaction (b) is not able to rise because the inactivation of the enzyme (by cyanide or low temperature) prevents it. As a result, the concentration of phosphoglyceric acid and of R will be lowered. If it is assumed that RH_2 will not be adsorbed at the chlorophyll, then the percentage of the chlorophyll surface not in contact with R and phosphoglyceric acid will increase as the light intensity surpasses the minimum value necessary to reach saturation. If, however, RH_2 stays adsorbed at the chlorophyll until it is chemically changed into the photo-

sensitive phosphoglyceric acid or until it is removed by oxidation (respiration), then the surplus of RH_2 will, like a narcotic cover, keep idle a part of the chlorophyll surface, and thus temporarily lower the rate below the final saturation rate. In the case of green plants, no fluorescence change would be connected with the inhibition caused by RH_2 . The actually occurring fluorescence rise in green plants in presence of oxygen is caused by the formation of the natural narcotic which is formed as soon as a part of the chlorophyll surface becomes available for photooxidation. Idling of the photosynthetic apparatus caused by a surplus of RH_2 would not be observed because it will only delay for a short time the formation of the natural narcotic. The removal of RH_2 by respiration is relatively slow and, therefore, some surplus of RH_2 is always present when the enzyme of reaction (b) is responsible for the saturation limitation.¹ The same way of emptying out a part of the chlorophyll surface will occur if the saturation is caused by carbon dioxide limitations.

We now come back to the fluorescence phenomena in diatoms. The emptying out of the photosynthetic apparatus is entirely analogous to the one in green plants. However, in this case we cannot use the alternative assumption that RH_2 may be released from the chlorophyll or that it stays adsorbed. Only the latter assumption fits the observations in diatoms. RH_2 must stay at the chlorophyll and must have the same activator qualities as R. Thus a temporary surplus of RH_2 causes not only the same high fluorescence as the contact of chlorophyll with R, but in addition it reduces the photosynthetic rate in the way discussed above. We may point out that we are unable to give an unambiguous explanation of the observation that in absence of carbon dioxide the fluorescence yield stays constant and high at all intensities. No break of the fluorescence curve is observed at the light intensity which is just sufficient to compensate respiration. The respiration in diatoms is entirely different from the usual one in green plants. It is not

¹ We may remind the reader in this connection of the results of early experiments of McAlister (18) and of Franck, French & Puck (16) which originally induced us to claim that carbon dioxide (or a carbon dioxide complex, as the molecule which adds the carbon dioxide by carboxylation was called at that time) is reduced photochemically at the surface of the chlorophyll. A leaf is exposed to light beyond saturation intensity under carbon dioxide limitation until the steady state of fluorescence is reached, then suddenly such limitations are removed by quickly admitting more carbon dioxide. The result is a dip in the fluorescence intensity which sets in immediately (as quickly as the gas atmosphere can be changed, that is in a couple of seconds). The dip is not a secondary influence of carbon dioxide because it is absent when the carbon dioxide concentration is suddenly raised from a value above the one necessary to avoid carbon dioxide limitations to still higher values. The old explanation remains in principle unchanged; we have only to replace the general term carbon dioxide complex by the special one, phosphoglyceric acid. We regard the quick response of the chlorophyll fluorescence to the addition of carbon dioxide as a strong indication for the assumption that phosphoglyceric acid is directly reduced photochemically in contact with excited chlorophyll.

only much greater but has, in addition, a highly abnormal respiration quotient. The oxygen consumption is two to three times higher than the carbon dioxide production. Tentatively, until more experiments are made, one may infer that the diatoms studied by Wassink & Kersten (21) belong to those cells in which intermediate products of respiration are quickly reduced photosynthetically in the light before they have a chance to evolve carbon dioxide. In that case most of the chlorophyll may be covered by R and RH_2 at all light intensities.

A relatively large amount of space has been given to our interpretation of the relation between fluorescence and photosynthesis in diatoms because it has not been published elsewhere, and it seemed desirable to show that these phenomena are in accordance with the general pattern of our theory. Wassink (43) and Wassink & Kersten's (21) interpretations, on the other hand, are given in several papers and in a general review. It may, therefore, be justified to present Wassink's interpretations more briefly, together with a few critical remarks and to refer the reader to the original papers for more detailed information.

Wassink's interpretation is based on the assumption "that any quantum absorbed in any place can move freely in some form through the entire chromophyllin, having definite probabilities of reaching the energy acceptor and definite probabilities of being abolished in various ways, among others as chlorophyll fluorescence." The name, chromophyllin is given to the protein-dyestuff surface, including chlorophylls and fucoxanthin, etc. The energy acceptor is a substance $RHOH$ to be reduced to RH . The fact that the fluorescence yield in diatoms at low light intensity is higher than in green plants is explained by the assumption that a part of the chromophyllin surface in diatoms is inactivated, whereby the energy transfer to the acceptor is hampered. This inactivation of the surface attributed to the action of respiration products is regarded as irrelevant for the photosynthetic quantum yield measured at the same low intensities. The elimination of a part of the chromophyllin surface does not change the utilization of the energy for photochemistry as long as enough energy acceptors are available to accept the energy after arrival. Only the migration time is prolonged and, while it is assumed that the prolonged travel time will increase the chance of losing some quanta on the way by fluorescence emission, this does not matter as long as the loss is small. We have two main criticisms of these assumptions. The hypothesis of a free movement of excitation energy over the whole chromophyllin surface which is identical with the hypothesis of a very extended photosynthetic unit offers difficulties regarded as prohibitive by the reviewer. That will be discussed in the paragraphs on the *Photosynthetic unit* in a later section. But even if the unit theory were right, it is difficult to see why in diatoms an inactivation of a considerable part of the chromophyllin surface (enough to enhance the fluorescence yield by a factor of about 3) will not influence the photosynthetic quantum yield, while in green plants an inactivation of a smaller part (estimated from the

smaller enhancement of fluorescence) of the surface brought about by an addition of urethane in low concentration will reduce the quantum yield considerably at the lowest light intensities. Wassink (21) explains the lowering of the fluorescence yield at saturation observed in presence of cyanide or at low temperature and the time dependency of this effect, as well as the corresponding increase of the saturation rate, by the hypothesis that cyanide poisons the oxygen-liberating catalyst and low temperature makes this enzyme inefficient. That prolongs the lifetime of the photoperoxides and makes it possible for them to react with the inhibitor and to remove it by oxidation. This explanation is in contradiction to statements by Franck & Herzfeld (44), Gaffron (45), and others that the cyanide limitation of photosynthesis is imposed by the sensitivity to this poison of the enzyme involved in the carboxylation process. To the old evidence speaking in favor of the latter hypothesis so much new experience has been added by the work with C^{14} (cf. the review of Fager & Gaffron in this issue) that Wassink's hypothesis can hardly be defended. Several other hypotheses are introduced by Wassink (43), among them the assumption that the energy acceptor RHOH might be oxidized to ROH and that the latter is the better energy acceptor. It would take more space than is available to give a detailed discussion of these assumptions, and the reader must therefore be referred to the original papers.

DIFFERENTIATION BETWEEN LIMITATIONS OF PHOTOSYNTHESIS CAUSED BY POISONS OR BY NARCOTICS

Poisons, by inactivating enzymes, have a strong influence on the saturation rate of photosynthesis while they do not change the rates if light is the rate-limiting factor, i.e., at low intensities. On the other hand, narcotics are supposed to cover surfaces. The covering of the chlorophyll surface will reduce the rate of photochemical reactions. Therefore, the rates will be lowered at low light intensities. If the surface activity of the narcotic is entirely unspecific, it is supposed to cover the same percentage of the protein chlorophyll surface as of the enzyme-protein complexes. In that case the photosynthetic rates should be influenced equally at all light intensities. These generally accepted principles are certainly sound but they are oversimplified and it is sometimes not easy by applying these principles to come to a clear-cut decision as to whether a certain substance acts as a narcotic or as a poison. The influence of a narcotic may deviate from the ideal one for different reasons. One of them is that the narcotic action may not be entirely unspecific. It might have a stronger influence on the chlorophyll surface or on the enzyme complexes. But even if the narcotic is entirely unspecific, its relative effect on chlorophyll and enzyme surfaces will be influenced by the competition of other substances for adsorption on the surfaces. We mentioned above the case of urethane in green plants and in diatoms. In green plants the substances to be reduced at the surface of chlorophyll are weakly adsorbed and, therefore, offer little competition to

narcotics. In diatoms, however, these substances seem to be strongly adsorbed and prevent the urethane from settling down on the chlorophyll surface as long as their concentration is high—meaning, at low light intensities. The result is that in diatoms the addition of urethane causes changes of the saturation curves which simulate the action of an enzymatic poison. The only criterion which proves that a substance acts like a narcotic is the observation that the slope of the rate curve at low intensities is lowered. Whether it is lowered less or more than the saturation rate is of secondary importance. However, even if a substance seems to act like a narcotic, the possibility exists that the action is a secondary one. The influence of hydroxylamine, and perhaps of *o*-phenanthraline, on the photosynthetic rate curve falls in this category. These substances are known to be poisons of the enzyme which liberate oxygen from the photoperoxides. Still their influence often looks like that of an ideal narcotic. In many plants the oxygen-liberating enzyme is poisoned by a metabolic product and becomes reactivated by the partial removal of that poison by a photosynthetic product (apparently it is oxidized by the photoperoxides). The effect is that the percentage of that enzyme which is active becomes proportional to the rate of photosynthesis. It is low at low light intensities and the highest percentage is active at saturation. If now an additional poison like hydroxylamine is added (whose concentration is independent of the light intensity), it will inactivate, at each light intensity, a constant part of the enzyme left active by the metabolic poison. Thus hydroxylamine and other poisons of the oxygen-liberating enzyme will reduce the rates by the same factor at high and low intensities. These considerations may have to be taken into account in the interpretation of papers of Macdowall (46) and of Holt, Brooks & Arnold (47). Macdowall's paper contains careful studies of the influence which known inhibitors of photosynthesis have on the rates of reduction of Hill reagents by chloroplasts. The main interest of this paper lies in the fact that some poisons which specifically inhibit photosynthesis have practically no influence on the rate of reduction of Hill reagents. Cyanide was again found to belong to this group. This result is in agreement with Hill's (7) and French & Holt's (48) observations. It is further in good agreement with the result showing that cyanide is a specific poison of the carboxylation reaction of photosynthesis. On the other hand, *o*-phenanthraline behaves like a narcotic. This result cannot be explained in the way described above because it is difficult to imagine that the concentration of active oxygen-liberating enzymes would be proportional to the rate of the reduction of the Hill reagent, indophenol, which was studied in these experiments. Other indirect actions are, however, quite possible; simultaneous studies of the chlorophyll fluorescence would be of interest in this respect. In other cases where inhibitory influences have been found to be stronger at low intensities than at high ones, the classification of the inhibitors seems doubtful because the measurements have, in these cases, not been extended to low enough intensities.

Holt, Brooks & Arnold (47) studied the inhibiting effect of irradiation with the line 2537 on photosynthesis in green algae and on reduction of Hill reagents of chloroplasts. The main result is that photosynthesis and photo-reduction in algae, as well as the reduction of Hill reagents, are equally inhibited by the preceding irradiation with ultraviolet light. The inhibition by a given dosage of light is the same for all light intensities and its dependence upon radiation time indicates a first order reaction with some deviations for short exposures. The deviations show that an effect of a higher order is superimposed. Of special interest in connection with the topics of this paragraph is the fact that the inhibition influences the photochemical reactions to the same percentage at saturation intensity as at very low intensities. Furthermore, if a certain concentration of hydroxylamine or of cyanide reduces the photosynthetic saturation rate of non-irradiated algae to one-half, it has the same influence on a sample of algae whose activity was already reduced to half the rate by ultraviolet irradiation. The latter result can be deduced from the former one, and both indicate that the inhibition by ultraviolet light acts very similarly to the inhibition caused by an "ideal narcotic." The observation indicates that the protein chlorophyll complex has the same sensitivity to ultraviolet light as the enzyme protein system responsible for saturation. Maybe both make use of the same protein.

CHEMICAL KINETICS

Photosynthetic unit.—The idea that several thousand chlorophyll molecules cooperate to deliver the energy which one of them has absorbed to a spot where it can be used for the reduction of carbon dioxide resulted from Emerson & Arnold's (6) measurements of photosynthesis with flash illumination. They concluded that the number of cooperating chlorophyll molecules would be about 2000. The idea gained support from Gaffron & Wohl's (49) calculation that the photosynthetic saturation curve, measured under optimal conditions, can be well understood if the concept of the photosynthetic unit is used. If the chlorophyll molecules do not cooperate, a Blackman period of the order of magnitude of a score of seconds would be expected, while actually the duration of that period as measured by Emerson & Arnold (6) is 1/100th of a second. Again, the cooperation of about 2000 molecules would eliminate the contradiction. Gaffron & Wohl (49) introduced the concrete picture of the energy migrating through several thousand chlorophyll molecules by exciton movements. If this assumption were true, the spectrum of the chlorophyll in the plants would show corresponding changes (see previous section). Förster (37), therefore, introduced the assumption that the energy transfer would be slow enough to avoid this difficulty, and calculated that during the normal lifetime of an excited chlorophyll molecule the energy might migrate through about 1000 of them at the concentration of chlorophyll found in chloroplasts. The actual lifetime of the fluorescent excited state of chlorophyll in plants is, however, 1/1000th of the normal

one as the low fluorescent yield proves. Thus, the energy can only migrate through a few molecules during the actual lifetime. However, the question arises as to whether the energy may not migrate after it is transferred to the metastable state by internal conversion. We must therefore analyze this possibility.

The lifetime of the metastable state of chlorophyll is not known, but even if it should be much smaller than the value set as the upper limit by Livingston (27) $1/100$ th of a second, there would be no difficulty in assuming that the triplet state excitation energy might migrate through thousands of chlorophyll molecules. The time is still long enough even if we take into account the fact that the migration time must be much smaller than the lifetime (for instance, by a factor 20), in order to avoid too high radiation losses. However, the travelling time must be reduced by another much higher factor because of the necessity of avoiding great losses of excitation energy of the triplet state by collisions with substances which make use of the energy for chemical purposes or transform it into heat. Since the triplet state has an excessively high sensitivity to impacts with oxygen, we take only these into account. Data on the sensitivity of the triplet state of chlorophyll to oxygen are not available, but we can make use of the fact that in dyes the quenching yield per impact during the lifetime of the excited state is the same in the lowest excited singlet state as in the triplet state. Each 200 impacts is a quenching impact for both excited states. The quenching yield for chlorophyll for the lowest singlet state is known, and we use the same value for the metastable state.

Tamiya & Huzisige (50) have recently very carefully measured the influence of oxygen on the rate of photosynthesis and concluded that no measurable influence on the rates at low light intensity can exist between the rate measured in nitrogen containing a few tenths of a per cent of oxygen and in an atmosphere of pure oxygen. Tamiya's methods permit the observation of changes smaller than 4 per cent. We, therefore, base our calculation of the maximum travelling time on the premise that not more than 4 per cent loss from quenching impacts with oxygen shall occur during the traveling periods when this gas is dissolved in water in equilibrium with 1 atm. of oxygen. We assume further, for reasons discussed in the preceding section, that all chlorophyll molecules are accessible to the oxygen. The result of the calculation on the basis of the assumptions and restrictions outlined above is that the travelling time must not be greater than 10^{-8} sec. If the degree of overlapping between emission and absorption spectrum for the triplet-singlet transition were known, a rigid calculation could be made of the number of chlorophyll molecules through which the energy may migrate in 10^{-8} sec. Since the spectra are unknown, we are forced to rely on estimates. Application of the so-called Franck-Condon principle to the transitions of the triplet-singlet system leaves little doubt that the overlapping of the spectra must be much less pronounced than for the singlet-singlet system. That gives a much smaller migration velocity for the energy of the excited triplet state than for the singlet state. The factor may lie

between 10 and 1000. Since, according to Förster (37) the energy of the lowest singlet state can travel through 1000 chlorophyll molecules in 10^{-8} sec., the triplet energy might pass through 100 chlorophyll molecules but probably through a much smaller number in this same time. The result is not encouraging for the assumption of a photosynthetic unit of 2000 chlorophyll molecules. While the preceding calculation is not better than a good estimate, it may add to the other evidence against the hypothesis of the unit theory which still has many adherents.

Space does not permit an elaborate discussion of all the other evidence against the unit theory on this occasion (51). We confine ourselves to enumerating: The dark period of 1/100th second is only one of the three which are known to be responsible for saturation under different external conditions. The two other dark periods have a duration between 20 sec. and 1 min. in accord with calculations not based on a unit theory. The period of 1/100th of a second can be explained without the unit by the assumption that it is the "working period" of an enzyme engaged in stabilizing short-lived photochemical products. The maximum photosynthetic gas exchange produced by a flash of very short duration, in that case, becomes equal to the number of enzyme molecules available for this particular reaction.

Two papers have appeared recently on flash measurements. Tamiya & Chiba (52) studied the yield per flash as a function of the temperature, and observed that it is lowered at low intensity. They believe that this result is in contradiction to the explanation just mentioned. However, that is not the case, as follows from a paper by Weller & Franck (53). They used strong light flashes of a longer duration than the one used by Tamiya & Chiba and under these conditions the maximum flash yield becomes independent of temperature. The flashes were, of course, shorter than 1/100th of a second, but at low temperature where diffusion becomes slow the flashes have to be longer than at high temperature to permit the enzymes to reach the photoproducts before losses occur on account of their instability.

The other paper of considerable interest contains observations of Clendenning & Ehrmantraut (54) showing that the period of 1/100th of a second is responsible for saturation of the process of reduction of Hill reagents by chloroplasts, and that the saturation rate of these reactions, under favorable conditions, can be equal to the saturation rate of photosynthesis at room temperature and in absence of carbon dioxide limitation. These results show that the particular enzymatic reaction is directly connected with the unspecific photochemical reactions of the chlorophyll in the chloroplasts.

INFLUENCE OF OXYGEN ON THE PHOTOSYNTHETIC SATURATION RATE

The inhibiting influence of oxygen on the photosynthetic saturation rate is of special interest because practically all photochemical reactions sensitized by chlorophyll *in vitro* are oxidation reactions in contrast to the reduction reactions of photosynthesis and of chloroplasts. However, a careful examination of many chlorophyll sensitized oxidation reactions has shown

that this contradiction is not a real one. There is a general agreement that the primary process in these reactions is a mobilization of hydrogen atoms. These react with molecular oxygen and form hydrogen peroxide, or the hydrogen peroxide radical HO_2 , which in turn oxidizes the substrate [cf. for instance, Weiss (55); Franck & Livingston (22)]. There are indications that chlorophyll enters into the chemical reactions either as intermediary hydrogen acceptor or donor. The photochemical part of photooxidation sensitized by chlorophyll is, in other words, a reduction of molecular oxygen by hydrogen atoms made available photochemically by a process for which the light-excited chlorophyll provides the energy.

The inhibiting influence of oxygen on the photosynthetic saturation rate was first observed by Warburg (3). The saturation rate in an atmosphere containing 0.03 per cent carbon dioxide will be lowered by about 30 to 40 per cent if the partial pressure of oxygen in the gas is raised from 1/100 atm. to 1 atm. Several observers have found that the inhibiting influence is much smaller if the carbon dioxide supply is high, and that oxygen had no influence at light intensities considerably below that necessary for saturation. The conclusion to which these observations have led is that oxygen competes with the substances to be reduced in photosynthesis for the hydrogen atoms provided by the photolysis sensitized by chlorophyll. Oxygen is successful in this competition only if the concentration of the substances to be reduced becomes small; i.e., in the neighborhood of saturation. But it is not possible to assume that the lowering of the saturation rate by 40 per cent is a direct measure of the rate of hydrogen peroxide formation. That would give too much hydrogen peroxide for the survival of the plant even if catalase is present. Another contradiction to this assumption stems from direct observations of oxygen consumption by plants carrying out photooxidation in the absence of carbon dioxide. Under these conditions the rate of photooxidation in air at saturation is at least 20 times smaller than would be expected from the inhibiting influence of oxygen (56). These two reasons in connection with fluorescence observations have, among others, forced the present reviewer to the assumption that, under conditions favorable to photooxidation, an expendable substance (sugar) is attacked and produces an inhibiting substance, the so-called natural narcotic. The part of the chlorophyll covered by this natural narcotic becomes inactive, for photosynthesis as well as for photooxidation. The extent of this surface cover adjusts itself to the prevailing conditions. Myers & Burr (57), who studied photooxidation processes under somewhat different conditions, also came to the conclusion that an inhibitor must be made by photooxidation.

Two new and important contributions on the oxygen influence have recently been published. Tamiya & Huzisige (50) measured the oxygen influence on the saturation rate in *Chlorella* under a variety of conditions. Their experiments are so extensive and accurate that now real quantitative data are available on this effect. However, the authors explain their results in a quite different, even though formally similar, manner from the one preferred by the writer of this survey. Their main conclusion is that molecu-

lar oxygen may prevent the carboxylation by competing with carbon dioxide for its place on the enzyme involved. This competition is assumed to be similar to that between oxygen and carbon monoxide for hemoglobin.

New experiments made recently by Mehler (58) give clear indications against Tamiya's hypothesis and in favor of our point of view. The procedure for these measurements is discussed in the report of Gaffron & Fager. Its principles are as follows: Mehler's method avoids competition between oxygen and substances which are reduced in normal photosynthesis by using chloroplasts in which the latter compounds are absent. He adds substances in abundance which remove hydrogen peroxide quickly in known reactions. Thus the rate of oxygen uptake during illumination becomes a measure of the hydrogen peroxide formation. The high rate of oxygen uptake is, according to our view, an indication that no narcotic layer interferes with the hydrogen peroxide formation, and that is expected because no reason exists for assuming that the substance which is oxidized in Mehler's system should have narcotic qualities.

INDUCTION PERIODS OF PHOTOSYNTHESIS

In previous sections examples have been given which show that the relation between chlorophyll fluorescence and photosynthesis are by no means simple, and it was also mentioned that the variability of fluorescence yields and rates is the greatest during the photosynthetic induction period. It soon became evident that this bewildering multiplicity of phenomena was caused by the dependence of the induction phenomena upon metabolic factors, as well as upon external conditions, including the previous treatment of the plant material. In such a complex situation it is certain that new observations will involve modifications or supplementation of our theoretical picture. At present we can only outline some of the older observations and interpretations and then discuss some new results and their probable significance.

The main factors which influence the induction phenomena are cell density, degree of aeration, culture condition, and the duration of the preceding dark period. If the plant material is multicellular, such as relatively thick leaves, or if old and dense cultures of unicellular plants are used, or if young cultures are not well aerated but are kept for a while under partial or total anaerobicity, then the induction phenomena observed by the time course of fluorescence or by oxygen production are much more pronounced than in young, well aerated, thin cultures. This was interpreted by Blinks & Skow (59) as an indication that in the dark period preceding the irradiation an unspecified inhibition develops. Franck and co-workers (42) concluded that the inhibition under these conditions was due to the accumulation of a metabolic poison in the dark which specifically poisons the oxygen-liberating enzyme. As a result of the inactivity of this enzyme, the photoperoxides produced by illumination will start the chain of events, sugar oxidation, leading to the formation of a layer of the natural narcotic and, as a result, all photochemical activity will be reduced. This short

period is followed by a longer one, which may last a couple of minutes, in which the photosynthetic rate rises until it reaches its final steady state. According to our interpretation, the poison level will be lowered during the recovery period from its dark concentration to its final light concentration, by an oxidation process involving the photoperoxides. Correspondingly the oxygen-liberating enzyme will be reactivated. Since the photoperoxides are photosynthetic products, the poison level and the activity of the enzyme adjust themselves to the photosynthetic rate. Any sudden change of the degree of light limitation or carbon dioxide limitation will cause the appearance of new induction phenomena. This self-adjustment of the activity of this enzyme causes the rates of carbon dioxide consumption and of oxygen evolution to change in a parallel manner during the induction period, the quotient, therefore, remaining normal. The time course of fluorescence intensities runs anti-parallel to both oxygen and carbon dioxide rate curves. This interpretation is consistent with a great number of observations. However, it has to be changed in one essential point, which will be taken up after the induction phenomena in better aerated plants have been discussed.

Mono-cellular green algae, grown and aerated in the usual way, and used in concentrations standard for manometric measurements, show induction phenomena which are less pronounced in respect to the rate of oxygen development and to fluorescence anomalies than leaves. The main reason for this difference must be a much smaller concentration of the metabolic product which poisons the oxygen-liberating enzyme.²

Evidence that under these conditions the induction phenomena of the carbon dioxide uptake are different from those for oxygen evolution became well known as a result of Emerson's (61, 62) work on quantum yields. We will come back to this point in the last section on QUANTUM YIELDS. However, the same phenomena can be seen quite clearly in some of the curves of McAlister & Myers (18) which permit following simultaneously the time courses of carbon dioxide uptake and of fluorescence. The fluorescence reaches its steady state intensity quickly, while the rate of carbon dioxide uptake rises for a much longer time until it reaches its normal level. This phenomenon is especially obvious in the few curves presented in McAlister & Myers' (18) paper where low light intensity was used. The significance of these observations was overlooked and not taken into account in the older theoretical considerations of the reviewer.

Blinks & Skow (59) have observed with the oxygen electrode, and Shiau

² Experiments with algae kept under strict anaerobic conditions have shown that anaerobicity has very little effect on the photosynthetic saturation rate, provided that the cell concentration is exceedingly small, but if the conventional concentrations are used, a few hours of anaerobicity inactivate the oxygen-liberating enzyme to such a degree that the saturation rate may be a thousand times smaller than the normal one. These experiments, supplemented by others, indicate that the poisonous metabolic product is a water-soluble substance whose penetration to the chloroplasts is accelerated by anaerobicity (60).

& Franck (17) with fluorescence measurements that if great care is taken to aerate the cells sufficiently or if very young and thin cultures are used, no induction at all is observed for the oxygen evolution and for the fluorescence. Both groups came to the erroneous conclusion that there would also be no induction phenomena for the carbon dioxide uptake under these conditions [cf. Warburg & Burk's (30) treatment of algae for measurements of quantum yield (next section)]. We now are aware from the results obtained with C^{14} that to reach the steady state of carbon dioxide uptake a suitable concentration of a compound able to add carbon dioxide has to be built up during the illumination period. Since, after a long dark period, the necessary reduced C_2 compound is not present, there will always be an induction period for carbon dioxide uptake equal, in its duration, to the time required by photochemical and dark reactions for the proper concentration of the reduced carbon dioxide acceptor. On the other hand, if the precursor, which is transformed photochemically into the carbon dioxide acceptor, is present, the oxygen evolution accompanying its reduction and the changes in fluorescence intensity will be normal, provided the activity of the oxygen-evolving enzyme is not coupled with the rate of carbon dioxide uptake. But even under conditions where, through poisoning of the oxygen-liberating enzyme, the induction phenomena of the rate curve of carbon dioxide consumption are coupled with anomalies in the curves for fluorescence and oxygen evolution some changes are necessary in our earlier explanation of the induction period. Apparently the adjustment of the activity of the oxygen-liberating enzyme to the photosynthetic over-all rate is, though a much faster process yet similar to the photochemical building-up of the steady state concentration of the reduced carbon dioxide acceptor. It is the latter reaction which, in each case, is responsible for the long, drawn-out induction phenomena. The difference between well and badly aerated plants is, then, that in the latter case the induction anomalies of carbon dioxide uptake are tied up with the transient anomalies of oxygen production and fluorescence intensities. The conception of how this coupling is achieved remains unaltered.

Van der Veen (63) has recently studied induction phenomena using the technique of Aufdemgarten (64). This technique, which depends upon changes in the resistance of a heated wire due to changes in the carbon dioxide content of the gas flowing past the wire, makes it possible to register, without too much delay, the changes in the rate of carbon dioxide consumption which occur during photosynthesis. The plant material used consisted either of leaves or needles of trees or of a layer of tightly packed algae deposited on the surface of a metal plate. Therefore, the conditions were such that during illumination periods the oxygen-liberating enzyme must become activated proportionally to the photosynthetic rate, and the course of the rates of oxygen production and of carbon dioxide uptake are supposed to run parallel.

Figure 4 shows an example of the rate curve of carbon dioxide uptake as measured by van der Veen (63) at high light intensity. The results are identical with those of Aufdemgarten (64). By employing a hydrogen

atmosphere containing a few tenths of a per cent of oxygen and by removing the carbon dioxide before passing the gas over the hot wire, van der Veen was able to use the apparatus also in measurements of the rate of oxygen production. The rate curve of oxygen production measured by him looks entirely like the curve for the carbon dioxide uptake. Curves identical in shape (but not in rates) with the observed curve for oxygen production have been found by Franck, Pringsheim & Lad (60) who studied oxygen production by algae under severe anaerobicity and used a quite different observation method.

Van der Veen (63) studied especially the first maximum of the time course

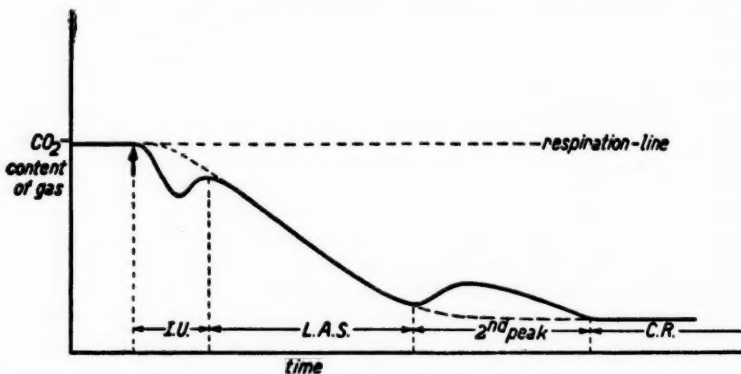


FIG. 4. Van der Veen. Normal induction curve for leaves in 3.65 per cent carbon dioxide.

of carbon dioxide uptake. At a temperature of 0°C , only this first maximum, called by him "initial uptake," remains. The so-called adaptation part of the curve is no longer visible. Van der Veen was also able to observe the initial uptake at room temperature by exposing the plant material to a temperature of 48 to 50°C . for a few minutes. Such pre-treatment entirely suppresses normal photosynthesis, probably by destroying an enzyme, but the initial uptake remains easily observable even though it is apparently somewhat reduced. Another change in the initial uptake resulting from the pre-treatment is still more interesting. In such heat pre-treated plants, the carbon dioxide absorbed during the initial uptake is held in the plant as long as the illumination period lasts and is released again as soon as the light is turned off. Equally important is the observation that this reversible initial uptake is not accompanied by any production or consumption of oxygen. There are also indications that the normal initial uptake in non-treated plants involves a potentially reversible reaction which becomes more important when the temperature is lowered.

The explanation given by van der Veen (63) for the adaptation part of the induction curve is, in principle, identical with the proposal presented

in this review. The author assumes that a substance must be made by the light which is needed for the adaptation to the final rate. He mentions further that the substance must play a role in Calvin & Benson's scheme of photosynthesis. However, his scheme and the one presented here are very different in their general conception. Van der Veen's scheme is based on a combination of Kok's (66) ideas that energy-rich phosphate bonds are made photochemically and that their energy is used directly for dark reduction of carbon dioxide, with the concept that the only photochemical reaction is the production of a general reducing agent. The same scheme is used for the explanation of the initial uptake. We maintain that the adaptation part of the induction period is the time necessary to accumulate a sufficient concentration of reduced C_2 compound to support the steady state production of phosphoglyceric acid. If some C_2 compound is assumed to be present initially, its reduction process, accompanied by oxygen evolution, will start as soon as the light is turned on. However, after a certain time which, according to experimental evidence, is about 20 sec., the supply of non-reduced C_2 compound will run low because it is not replenished quickly enough. Therefore, the rate of oxygen production will temporarily go down. From this minimum rate both oxygen evolution and carbon dioxide absorption will rise slowly to their final rates for steady state photosynthesis. To explain this, we have to introduce an assumption about the way in which the C_2 compound is made. We know that it is a product of photosynthesis and of dark reactions. We believe that it is made photosynthetically before a stable sugar is made and after the formation of phosphoglyceric acid. The concentration of such a substance will, of course, be small at the first part of the irradiation period and, therefore, a temporary lack of C_2 compound may result when the old supply is used up and before sufficient of the new is made. Just as the oxygen production rises quickly and falls again, the carbon dioxide uptake (connected with the reaction between the reduced C_2 compound and carbon dioxide to form phosphoglyceric acid) will rise and fall again. It is expected that the time course of CO_2 uptake may lag a little behind that of oxygen evolution. According to a result of Aufdemgarten (64), cyanide strongly diminishes the phenomenon of initial uptake of carbon dioxide. If applied in small concentrations, cyanide may be expected to flatten the maximum of the initial uptake and prolong it because the enzyme involved in the carboxylation reaction is partially poisoned. Thus, the initial uptake might be masked by the general rise of rates during the adaptation period. At low temperatures ($0^\circ C.$) the initial uptake is the only part of the induction curve which remains. We interpret this by the assumption that the process of replenishing the non-reduced C_2 compound is slowed down exceedingly at low temperature because of the dark reactions involved. This assumption also explains the observation that after the first observation of the initial uptake at low temperature one has to allow a dark period of at least 15 min. before another fully developed initial uptake can be observed.

The occurrence of a reversible initial uptake in heat-treated plants is of

special interest, for it is not accompanied by oxygen evolution. The fact that carbon dioxide is taken up in the light and is retained as long as the illumination lasts can hardly be explained in any other way than by the assumption that some compound, perhaps C_2 , is converted photochemically into a form which can add carbon dioxide. The process does not involve a reduction because no oxygen evolution is connected with it. The addition compound is unstable and easily loses carbon dioxide, the acceptor substance quickly reverting to its stable form. This uptake and release of carbon dioxide continues as long as the illumination continues. The simplest assumption is to regard the unstable compound as a tautomer of the stable.

In normal plants the formation of the unstable addition product of carbon dioxide is followed by its photochemical reduction and transformation into phosphoglycerate; in heat treated plants the enzyme involved in this process is lacking for they show neither photosynthetic oxygen production nor permanent carbon dioxide fixation.

Another important contribution to the problem of induction periods may be mentioned briefly. Ehrmantraut & Clendenning (54) have found that no induction losses occur for the photochemical reduction of quinone in cells or chloroplasts. This is as expected because no adaptation process is needed. There is also no reason why the oxygen-liberating enzyme should be poisoned because the dark metabolism providing the poison is, if present, certainly much weaker in chloroplasts than in normal living cells, and quinone, which was used by the authors as a Hill reagent, is known to stop all dark metabolic processes.

REMARKS ON QUANTUM YIELDS

It would not be justified to write a critical review on the physical background of photosynthesis without discussing the basic problem of photochemical quantum yields. However, the discussion can be brief because the readers know about the existing controversy and its scope. Warburg & Burk (30) and others maintain that the quantum yield of photosynthesis is $\frac{1}{4}$ —the classical value of Warburg's earlier results—while a number of other investigations carried out, mostly by scientists in the United States, gave the value $\frac{1}{3}$ as optimal observed yield. Among the latter group the work of Emerson *et al.* (61, 62) has to be mentioned especially, because the authors used exactly the same procedure as Warburg. They used the manometric technique, measured the yield in the neighborhood of the compensation point of photosynthesis, and used a great surplus of algal cells to achieve total absorption of the red light used for the measurements. (In a number of experiments a general weak irradiation with white light was superimposed.) In Warburg & Burk's apparatus only 5 per cent of the algae are exposed to the red light at each moment. However, the algae are whirled around quickly and thus all of them pass periodically through the region irradiated with red light. In effect the algae are thus exposed to a kind of flash illumination. In his last measurements, Emerson copied Warburg's procedure as exactly

as possible. Agreements between Warburg and Emerson's results are reached only insofar as both find that the quantum yield is 1/10th if the algae are suspended in carbonate buffers. For the measurements in acid suspensions the old divergence of results still exists. Emerson (62) surveyed recently all the transient anomalies of gas exchange at the beginning and end of illumination periods and pointed out that they can be sufficient to falsify the calculated results by 100 per cent or more if the quantum yield calculations are based on the total difference of manometer readings of 10 min. irradiation and 10 min. of darkness. In this connection, the occurrence of a strong outburst of carbon dioxide at the beginning of the irradiation and a slower reversal of this process in the following dark period has to be taken into account. There is no doubt that these effects observed by Emerson & Lewis (61) are real. Blinks & Skow (67) observed quite similar phenomena with the potentiometric method. Furthermore, the time course of the carbon dioxide evolution as observed by Emerson & Lewis (61) is very similar to that of fluorescence anomalies in algae at the beginning of irradiation periods. The effect may be explained by an oxidative decarboxylation which may be a part of the oxidation reaction of the induction period which is the consequence of a temporary inactivity of the oxygen-liberating enzyme. However, the "Emerson effect" cannot be responsible for the more recent results of Warburg, Burk and co-workers because the carbon dioxide outburst just as the induction phenomena change with the degree of aeration of the algae during and before the measurements. Warburg & Burk pre-treat their algae by exposing them for hours to a violent bubbling of air (with added carbon dioxide) during illumination. According to a private communication by Emerson, that treatment gives stronger respiring cells and very much diminishes the strong outburst of carbon dioxide at the beginning of irradiation. However, even under these conditions certain anomalies at the start and end of the irradiation period remain and Emerson holds them, according to a personal communication, as responsible for Warburg's results. Again one cannot doubt that irregularities of the type which Emerson claims are real. There must be an induction phenomenon for the carbon dioxide uptake after a period of 10 min. darkness, and on the other hand there is found by many observers the so-called "pick-up" of carbon dioxide right after the light is turned off [McAlister (18); Aufdemgarten (64); van der Veen (63); see also Gaffron & Fager's report on the fixation of $C^{14}O_2$ after irradiation periods (11)]. Whether these irregularities will be strong or negligible under the conditions of the "flashing illumination" used by Warburg & Burk, and by Emerson, the reviewer is unable to say.

Kok (65, 66) and recently van der Veen (63) have reported that a four-quanta process may occur below or in the neighborhood of the compensation point but it is replaced by an eight-quanta process at higher light intensities. Kok (65) and Franck (68) have published attempts to reconcile the contradictory results by the assumption that under certain conditions products of respiration (68) or the energy gained by respiration (65, 66, 68) can be

used for photosynthetic purposes. There is, however, no sense in discussing here these theoretical considerations as long as the interpretations of the experimental results (not the observations themselves) are still so divergent. It must, however, be mentioned in this connection that measurements of Weigl (69) with C^{14} , and quite recently direct and careful measurements of respiration of different plants in the dark and in the light by Brown, Nier & Van Norman (70) have shown that in some plants respiration can be suppressed during illumination. Brown *et al.* (70), who carried out their measurements with the help of O^{16} and O^{18} [for details, see Gaffron & Fager's review (11)] have, however, not found such an effect with *Chlorella* algae, the test organisms of Warburg and Emerson. That does not exclude the possibility that *Chlorella* cultured and treated according to Warburg & Burk may show an interference of photosynthesis with respiration. According to a private communication by Brown, experiments to test this possibility are in progress.

Photoreduction needs ten quanta under optimal conditions to reduce one carbon dioxide molecule (71). Dr. Larsen from Norway, who made every effort while in van Niel's laboratory to break this record was, as he kindly informed us orally, quite unable to do so. This is of interest because the total energy stored chemically by the process of photoreduction is about zero. In this case, at least, nature, was not interested in performing the assimilation of carbon dioxide as cheaply as possible.

From the point of view of kinetics, one would wish that Warburg's quantum yield, one-fourth, would be the right one because it would be the simplest solution if the four hydrogen atoms which are needed for the reduction of carbon dioxide would be produced by four absorption acts. The photochemical part of photosynthesis might, however, not only consist in transfer of hydrogen gained by photolysis of water; there might be other steps by which light energy is stored chemically. If the interpretation given in the preceding section for van der Veen's (63) reversible carbon dioxide uptake is correct, one would be inclined to see in this observation an example for energy-storing photochemical steps other than reduction processes.

From the point of view of thermodynamics, it is difficult, according to our present knowledge, to understand how a four-quanta process will provide all the energy needed for reducing one carbon dioxide molecule. In this review, reasons have been given which practically force us to assume that not all of the energy of the red light quanta absorbed can be used for photosynthesis. The excited chlorophyll molecules lose energy by a transition into the lowest triplet state before the photochemical reactions occur. We do not know exactly the position of this metastable state but reasons have been mentioned for assuming that this electronic state lies 5 to 10 calories lower than the singlet state excited by the red light. In that case it is already an optimistic evaluation of the situation to say that 148 calories are available for carbon dioxide reduction if four quanta are absorbed. Of the 148 calories, an amount of 120 calories per M must be stored as chemical energy (free

energy stored in a one-carbon carbohydrate group). The remaining 28 calories must, then, cover all energy losses which will occur on the complicated pathway of photosynthesis. They include not only stabilization energy of four photochemical products but also all the losses connected with the enzymatic dark reactions, and finally the energy released in the process of liberation of oxygen out of photoperoxides. If the last mentioned item equals the energy dissipation if oxygen is liberated out of hydrogen peroxide, then it alone would be one and one-half times greater than the total energy available for heat losses.

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THE KINETICS AND CHEMISTRY OF PHOTOSYNTHESIS

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INTRODUCTION

Well over ten years ago it was firmly established that the process of photosynthesis consists of three main parts which can be roughly characterized as (a) the photochemical decomposition of water, (b) the evolution of oxygen, and (c) the reduction of carbon dioxide. This knowledge is based upon a vast amount of information [Rabinowitch (1)] concerning the kinetics of photosynthesis, photoreduction in bacteria, and the photochemical reactions of isolated chloroplasts. Part (a), the conversion of radiant to chemical energy which is unique in the living world, is discussed by J. Franck in another review in this volume. We will review the papers of the past year which have dealt with parts (b) and (c), especially the recent advances [Benson & Calvin (2)] in the understanding of part (c) which have resulted from the availability of carbon isotopes and their usefulness in tracing the transformations of carbon compounds. In doing so, we will attempt to show what they contribute toward answers to the following questions which seem basic to an understanding of the photosynthetic process.

(i) What, if any, direct effect does photosynthesis or light have on respiration? (ii) What are the intermediates between carbon dioxide and the first well-established product of photosynthesis, which in most cases is sucrose? (iii) What relation is there between dark uptake of carbon dioxide following illumination and photosynthetic uptake? (iv) How true is the oft proposed hypothesis that the only unique feature of photosynthesis is the direct reduction of hydrogen carriers by photolysis of water, these hydrogen carriers then accomplishing the reduction of carbon dioxide to sugar by reactions similar to those already known in plant and animal tissues? [van Niel (3)] (v) What sort of scheme for the photosynthetic process can be written on the basis of present knowledge? Is photosynthesis literally the reversal of respiration or glycolysis? (vi) What is the mechanism of oxygen evolution as shown by chloroplasts? (vii) What relation is there between the reductions performed by chloroplasts and those which occur in photosynthesis?

The answers to these questions are all more or less incomplete and for many of them we have, as yet, only faint indications of the direction in which research should proceed to obtain the necessary information.

It is obviously impossible to cover all papers adequately within the limits of such a review. A selection must be made and, as such a procedure involves individual judgment, it is fallible.

RESPIRATION DURING PHOTOSYNTHESIS

Whether the respiration of photosynthesizing cells is enhanced, inhibited, or unaffected during illumination is a question which has plagued the investigators of photosynthesis since the days of the very first quantitative experiments. When the algae *Chlorella* or *Scenedesmus* are used as objects of research, it has become common practice to correct for respiration as if the latter continued unchanged during periods of illumination. This procedure has not only given the most consistent results but is indeed indicated as being the correct one by some indirect experimental evidence [cf. (1), Warburg *et al.* (4), Davis (5)]. Since the discovery that decarboxylations can be reversed during ordinary respiratory metabolism, the problem of respiration versus photosynthesis has received renewed attention, particularly on the assumption that the photosynthetic assimilation of carbon is a reversal of respiratory or glycolytic reactions. When it was found that phosphoglyceric acid appears as an intermediate in photosynthesis, this assumption seemed almost a certainty. Further work has indicated, however, that the other compounds involved in respiration or glycolysis are not involved in photosynthesis. Nonetheless a strong, reciprocal influence between photosynthesis and respiration could reasonably be expected even if phosphoglyceric acid were the only intermediate common to both processes.

Direct evidence from oxygen isotopes.—All experiments which hitherto have seemed either to support or to deny a direct linkage between photosynthesis and respiration have been based on indirect evidence and deductions. A new method, the continuous analysis with the mass spectrograph of gases containing oxygen isotopes, has now provided direct evidence by allowing observation of oxygen uptake by the illuminated cells during a period of rapid production of oxygen from water by photosynthesis. Recent experiments [A. H. Brown, A. O. Nier, and R. W. Van Norman (personal communication)] in which air enriched with O^{18} was used as the source of respiratory oxygen, and normal water (O^{16}) as the source of photosynthetic oxygen, have given rather surprising answers. The relation between respiration and photosynthesis in various species is shown in Table I. The experiments with *Chlorella* show definitely that in ordinary nutrient media light has no effect whatsoever on the trend of respiration. The heavier isotope is taken up from the air at a constant rate regardless of the rate at which oxygen is produced photosynthetically from the water. This confirms the earlier conclusions based on indirect evidence. In a blue-green alga, *Anabaena*, quite the contrary is true. Such low light intensities as would produce a photosynthetic rate only three times as great as the respiratory rate are sufficient to stop completely the uptake of oxygen from the air. The leaves of higher plants, where complications could be expected due to delayed outward diffusion of photosynthetically-produced oxygen and its use in preference to that diffusing into the cell from outside, show no influence of light upon the utilization of external oxygen. This indicates that the inhibitions observed in some of the algae are not due to purely mechanical factors, such as diffusion.

Where an effect of light upon respiration would have been welcome for reasons connected with the measurements of quantum yields, namely, in *Chlorella*, (cf. Franck, this volume), it was absent. The next question is, of course, whether under conditions of high cell density and low pH, such as were used to obtain high quantum efficiencies, it is possible to modify the normal behavior of the algae and make their respiration sensitive to light.

Among the data of Table I there is none that indicates an increase of

TABLE I
PHOTO-INHIBITION OF RESPIRATION*

Species	Light intensity†	Per cent inhibition of respiration
<i>Chlorella pyrenoidosa</i>	1-11	0
<i>Chroococcus</i> sp.	1.5- 3	35
<i>Porphyra umbilicalis</i>	.2- 7.7	0- 59‡
<i>Ulva lactuca</i>	.3- 9.2	0- 96‡
<i>Cylindrospermum</i> (or <i>Anabaena</i> ?)	.8- 3	40-100‡
<i>Pinus mugo</i>	5	0
<i>Nicotiana</i> sp.		
<i>Spinacia oleracea</i>		
<i>Hordeum vulgare</i>		
<i>Metasequoia glyptost.</i>		

* Personal communication from A. H. Brown, A. O. Nier & R. W. Van Norman.

† Measured by the ratio, photosynthesis/respiration.

‡ Increases rapidly with increasing light intensity.

respiration in the light. From time to time the claim of a special light respiration is repeated in the botanical literature. It was long ago (6) demonstrated that the extra oxygen uptake can be explained as a pathological photooxidation which may even produce an enhanced respiration for a considerable period afterwards in the dark.

Respiration and protein synthesis.—Benson & Calvin (7) have suggested that light inhibits respiration in *Chlorella* and barley on the basis that intermediates in the tricarboxylic cycle, as represented by glutamic and isocitric acids, are not tagged during short periods of photosynthesis. Steward & Thompson (8) have criticized this conclusion and have suggested that the lack of tagging in Krebs cycle intermediates may be due to a change in the source of these compounds from sugar to glutamic acid. Low carbon dioxide pressure has been shown to stimulate protein synthesis in green cells, presumably by reducing the rate of carboxylation of pyruvic acid to oxaloacetic acid and substituting for it the deamination of glutamic acid to ketoglutaric acid. If photosynthesis maintains a low internal carbon dioxide pressure, this same substitution would occur and tracer would appear in protein before it appears in the Krebs cycle intermediates. This is consistent with the observation that the major portion of the insoluble products formed by

algae in short periods of photosynthesis is protein, and that glutamic acid acquires significant tracer only after longer times.

EXCHANGE REACTIONS AND ISOTOPE DISCRIMINATION

The apparently simple method of using tracers in metabolic studies is often severely handicapped by two types of side-reactions: exchange and discrimination.

Exchange reactions.—An example of the difficulties encountered due to possible exchange is furnished by the photoreduction with hydrogen in purple bacteria. It is easy to show that none of the deuterium gas used as tracer and absorbed by purple bacteria during photoreduction appears in the cell material. This apparently proves the validity of certain metabolic schemes, but it is very difficult to be sure that the result is not robbed of its significance by an exchange of hydrogen for deuterium during some intermediary steps of the process (Brown & Gafron, unpublished).

If Fig. 2 of Calvin *et al.* (9) represented the usual situation, one would be forced to assume that there is a rapid initial fixation of tracer carbon dioxide by exchange. This exchange is of such magnitude as to effectively double the rate of tracer uptake for the first 30 sec. It is obvious that if such conditions obtain, results of all short-term photosynthetic fixations must be suspected of being solely or partly the result of exchange. However, we (unpublished observations) have always observed that when total photosynthetic tracer fixation is plotted against time for periods of 10 to 120 sec. the points fall on a straight line which extrapolates to zero tracer at 5 ± 1 second. A purely mechanical lag of about 1 sec. would be expected due to time for mixing, diffusion, equilibration with carbon dioxide reservoirs in the cells, etc. The rest of the apparent lag can easily be accounted for by the loss of potential fixation due to the killing method (cf. section on DARK FIXATION FOLLOWING PHOTOSYNTHESIS).

We have also established (unpublished observations) that the indicated importance of phosphoglyceric acid is not due to an initial rapid exchange involving a "labilized" carboxyl group of phosphoglyceric acid. If the activity fixed in phosphoglyceric acid in periods of 10 to 40 sec. is plotted against time, the points fall on a straight line which extrapolates to 1 ± 0.5 second for zero activity in phosphoglyceric acid.¹ If a rapid initial exchange were the basis of the indicated importance of phosphoglyceric acid, the extrapolation would show a negative time for zero activity in this compound. The same type of proof shows that pyruvic acid is not labeled by exchange.

Isotope discrimination.—Discrimination against a tracer, for instance against C^{14} in the presence of excess C^{12} , may falsify the result of rate measurements of photosynthesis or respiration. Some data have been published on such discrimination effects in the use of carbon isotopes during photo-

¹ The difference in the times for zero activity (total counts and counts in phosphoglyceric acid) is a result of the steeper slope of the line for total photosynthetic fixation and the use of 10 sec. as the first point in both cases.

synthesis. Not only is there a discrimination in favor of the assimilation of the lighter isotope, as expected, but the effect is unexpectedly large and out of proportion for C^{14} as compared with C^{13} . From the work of Van Norman (10) we give the following average ratios for photosynthetic rates in *Chlorella* and barley at about 1000 foot candles.

$$C^{13}/C^{12}^* = 0.96; \quad C^{14}/C^{12}^* = 0.85; \quad C^{14}/C^{13}^* = 0.89$$

* These represent ratios of rates of utilization of the isotopes.

This seems to confirm some of the results obtained earlier by Weigl (11). Following up these experiments, Van Norman found indications that compounds formed during an immediately preceding period of photosynthesis are preferentially respired. The available data on discrimination, preferential respiration, etc., must at present be taken as preliminary, since the number of observations is small in relation to the intrinsic difficulty of this type of research.

INTERMEDIATES IN PHOTOSYNTHESIS

The history of photosynthetic experiments with carbon isotopes, which began in 1939 (12), shows a continuous retreat to shorter and shorter times of exposure to tracer during photosynthesis executed in the hope of getting away from the deceptive complications of secondary dark reactions. The first success came with the discovery that phosphoglyceric acid is practically the only stable substance containing labeled carbon after tagging periods of very short duration [(Calvin & Benson (13))].

During the past year this discovery has been confirmed and extended [Fager, Rosenberg & Gaffron (14); Fager & Rosenberg (15)]. Further, the formation of phosphoglyceric acid has been found to be the major fixation reaction occurring in the dark immediately after the light was turned off. This reaction shows considerable differences from the better studied reversible decarboxylations leading to malic or ketoglutaric acids (cf. *Effects of poisons* in next section).

Of all the other substances identified by the elegant method of radiochromatography after short tagging periods [Benson *et al.* (16)] only pyruvic acid, malic acid, and glycolic acid are at present suspected of a close relationship to the photosynthetic process. The other compounds which are found to be labeled appear to be precursors of fat, starch, and protein syntheses rather than intermediates in photosynthesis. It is a significant comment on the nature of the whole problem that after several years of intensive study only one compound, phosphoglyceric acid, has with any certainty been directly implicated in the process of photosynthesis. The formation of triose or triose-phosphate from phosphoglyceric acid, the next logical step toward hexose synthesis, seems also to have been assured [Aronoff & Vernon (17)]. Beyond the triose level the specific task of photosynthesis is ended and the synthesis of various sugars appears to follow the pathways already known from carbohydrate metabolism.

Experiments done with $C^{18}O_2$ and *Nitella* (18) early in the study of photo-

synthesis with carbon isotopes indicated that carbon dioxide fixation during photosynthesis occurred largely in the chloroplasts, while dark fixation was confined to the cytoplasm. More recent experiments with *Chara*, using $C^{14}O_2$, have given no evidence of such an effect. After exposures to tracer carbon dioxide for periods of 10 sec. to 25 min. in either the light or the dark, the fixation products were found not to be restricted to either the chloroplasts or the cytoplasm [K. A. Clendenning (personal communication)].

In the following sections, recent findings concerning the intermediates of photosynthesis will be discussed in detail.

Phosphoglyceric acid.—The original identification of phosphoglyceric acid was based upon its behavior on anion exchange resins and various other physical properties (13). Benson *et al.* (16) have presented three additional proofs of its identity: co-chromatography on paper with known labeled phosphoglyceric acid from yeast; fractional elution from anion exchange resins of the radioactive material with authentic carrier material; and a direct isolation of barium phosphoglycerate whose phosphorus analysis and molybdate-enhanced rotation are given as criteria for its identification. The amount of activity in the isolated barium salt allowed them to calculate that over 65 per cent of the radioactivity fixed in 5 sec. photosynthesis by *Scenedesmus* (10,000 foot candles) was in the form of phosphoglyceric acid. It has since been reported (9) that at this light intensity 87 per cent of all activity fixed is in phosphoglyceric acid, 10 per cent in pyruvic acid, and 3 per cent in malic acid.

We have corroborated these reports and have isolated by ion exchange fractionation macroscopic amounts of phosphoglyceric acid from *Scenedesmus* which had been exposed to tracer for illumination periods of 30 to 40 sec. (ca. 500 foot candles). Appropriate derivatives were prepared and analyses of these proved the identity of the compound (14, 15). Because, during the isolation, the major activity peak was always chosen, there is no question that phosphoglyceric acid was the only individual compound in which large amounts of tracer had been fixed. Both groups have presented evidence that at moderate to high light intensities phosphoglyceric acid is the compound whose relative tracer content decreases with time, whereas other cell constituents increase in tracer content relative to the total fixation. This is good evidence for the supposition that phosphoglyceric acid is indeed the first stable compound in which tracer is fixed. Similar results have been reported for tracer fixation in soy beans [Aronoff & Vernon (17, 19)], barley, and other plant materials.

Analysis of tracer fixation at low temperatures (2°C.) has shown that 2-phosphoglyceric acid is probably the first product (9). At normal temperatures (20°C.) this is rapidly converted into the 3-isomer so that the equilibrium mixture is what is usually isolated.

Work in both laboratories (9, 14) has established that the carboxyl group is labeled first and that within a relatively short time tracer appears in the α - and β -carbons of phosphoglyceric acid. In *Scenedesmus* phosphoglyceric

acid is uniformly labeled after 2 min. of photosynthesis at a light intensity of about 500 foot candles. Some early reports indicated that the α -carbon was labeled before the β -carbon, but recent publications (9) and experiments in our laboratory have shown that equal labeling of these two carbons in respect to time is the usual case.

In accordance with this, labeling in the hexoses, both free and resulting from sucrose hydrolysis, has been shown to occur in the 3,4 positions first, and then in the 1,6 and 2,5 positions in equal amounts [(9, 17); Gibbs (20)]. The distribution of tracer in glycolic acid (9), malic acid, alanine (20), glycine, and serine (21), after short periods of photosynthesis, is in complete agreement with the order of labeling for phosphoglyceric acid; i.e., the 3- and 4-carbon compounds have the most label in the carboxyl and the α - and β -carbons are equally labeled; the 2-carbon compounds are symmetrically labeled. Vittorio *et al.* (22) have shown that earlier reports that the 1,6 positions of the hexoses had the highest activity were apparently due to dilution of the label in positions 3,4 by $C^{12}O_2$ in the time elapsing between exposure to tracer and killing.

The first evidence that phosphoglyceric acid may be reduced photochemically (14) was presented by the marked difference in distribution of activity when algae, *Scenedesmus*, which had been illuminated in the absence of carbon dioxide and oxygen were allowed to fix tracer in the dark or in the light. In 60 sec. in the dark over 95 per cent of the activity fixed was in the form of phosphoglyceric acid and pyruvic acid; in 10 sec. in the light these two compounds represented only a little over 60 per cent of the total fixation. This was taken to indicate that fixation in the light involves transformation (reduction?) of phosphoglyceric acid in addition to carbon dioxide fixation. As further evidence, algae were illuminated in the absence of carbon dioxide and oxygen and allowed to fix tracer in the dark (95 per cent in phosphoglyceric acid and pyruvic acid), the unfixed tracer was washed out and part of the algae was illuminated a second time in carbon dioxide-free buffer while part was kept in the dark. After such treatment, the amount of tracer contained in phosphoglyceric acid in the part illuminated for 40 sec. was only 35 per cent of that found in phosphoglyceric acid in the dark control. The illumination did not change the total tracer content.

Pyruvic acid.—Tracer carbon seems to appear in pyruvic acid as soon as it does in phosphoglyceric acid (9, 14). It at first seemed plausible that pyruvic acid should be the initial product of carboxylation followed by phosphoglyceric acid. However, the ratio of activity fixed in phosphoglyceric acid to that fixed in pyruvic acid during photosynthesis contra-indicates this order (14). This ratio remains between 5:1 and 7:1 if the algae are killed when the light is turned off. However, if they are allowed to respire in the dark before killing for even so short a period as 20 sec., the ratio decreases to less than 4:1, indicating that the necessary enolase is probably present (the final equilibrium value should be phosphoglyceric acid/pyruvic acid = 3). If pyruvic acid preceded phosphoglyceric acid and the transformation

were the normal enzymatic one, this ratio could never exceed 3:1. Therefore, if pyruvic acid is involved in photosynthesis it probably follows phosphoglyceric in the sequence of compounds.

Wood (23) has pointed out that this is not a rigorous proof of the order in which these compounds become tagged and has suggested a mechanism for maintenance of the disequilibrium in terms of the structure of the cell. He, however, feels that the presently available evidence makes the precedence of phosphoglyceric acid the more plausible assumption. It is also possible that phosphoglyceric acid and pyruvic acid are derived from a common enzyme-complex precursor in a manner similar to that demonstrated for malic and oxaloacetic acids [Wood (24)].

Malic acid.—Calvin *et al.* (9) have reported that small percentages of tracer are fixed in malic acid during very short periods of photosynthesis (5 sec., 10,000 foot candles) and that with low light intensities (45 foot candles) this compound becomes the major site of tracer fixation. They at first considered it the likely result of a reductive carboxylation of pyruvic acid on the way to a 2-carbon acceptor for carbon-dioxide and thus as an intermediate in photosynthesis. However, using malonate as an inhibitor of malic acid synthesis and studying the rate of appearance of tracer in the α - and β -carbons of phosphoglyceric acid, they (25) have demonstrated that even when malic acid synthesis is inhibited to the extent of 70 to 80 per cent, tracer appears in the α - and β -carbons of phosphoglyceric acid at a rate comparable to that in unpoisoned controls. This would seem to indicate that malic acid is not directly involved in photosynthesis.

At low light intensities, the rate of respiration is nearly or quite equal to that of photosynthesis. Under such conditions, it is reasonable to regard the labeling of malic acid as the result of a competition between photochemical reduction of phosphoglyceric acid to triose and enzymatic transformation of this compound to pyruvic acid followed by reductive carboxylation to malic acid. At low light intensities the rate of malic acid formation may be nearly as great as the rate of phosphoglyceric acid formation and thus tracer may accumulate in malic acid and disappear from phosphoglyceric acid giving an incorrect impression of the importance of malic acid. The work at low light intensities (26) further suffers from the lack of an adequate dark control and the long period between exposure to light and killing.

Varner & Burrell (27) have reported that dark fixation in the succulent, *Bryophyllum*, results largely (75 to 85 per cent) in fixation in the carboxyl groups of organic acids, malic acid containing three to four times as much tracer as any other acid. Only 0.5 per cent is found in sugars and starch. However, when leaves which have fixed tracer in the dark are illuminated in the absence of tracer, a relatively rapid transformation of the acid to sugar and starch is effected. Fifty per cent or more of the tracer found in the sugars after such treatment is in the 3,4 positions. They suggested that if malic acid were a precursor to a 2-carbon carbon dioxide acceptor or the source of such a precursor, tracer should appear largely in the 2,5 positions of the sugar. However, Calvin *et al.* (9) have shown that by appropriate balancing

of a reverse Wood-Werkman reaction (decarboxylation) and the cycle which they have proposed, one could obtain a tracer distribution such as is reported.

Glycolic acid.—Benson *et al.* (7) report that tracer appears in glycolic acid after relatively short periods of photosynthesis, that it is apparently formed only in the light and disappears rapidly in the dark, that the relative amount fixed in it is increased with increasing oxygen pressure, and that illumination under aerobic conditions in the absence of carbon dioxide constitutes the optimal condition for appearance of tracer in both glycolic acid and glycine. These last effects, they suggest, may be the result of photooxidation of the 2-carbon carbon dioxide acceptor. If this suggestion is correct, it provides support for the postulation of glycolaldehyde, or a closely related compound, as the 2-carbon acceptor for carbon dioxide.

Both Ochoa (28) and Lipmann (29) have suggested that glycolic acid in the form of glycolyl phosphate may be the substance which is reductively carboxylated to form a derivative of the as yet unidentified hydroxy-pyruvic acid. If this suggestion is correct, glycolic acid is itself the 2-carbon acceptor for carbon dioxide.

In connection with glycolic acid, it is of interest that Burris & Tolbert (30) have found an enzyme in plants for the oxidation of this compound. This enzyme is present only in the green portions of plants and is absent from albino corn and etiolated plants. The latter contain enzymes for the oxidation of ascorbic, glyoxylic, and *L*-lactic acids so that the absence of the glycolic enzyme is not merely part of a general dearth of enzymes in etiolated plants. The glycolic enzyme is activated by illumination of etiolated plants, this activation being more rapid than chlorophyll formation. The relationship of these observations to the process of photosynthesis is at present unknown, but the obligate association of the enzyme with photosynthetically active materials is suggestive.

Other products of photosynthesis.—Aronoff & Vernon (17) have shown that, as might be expected, the formation of trioses precedes that of hexoses and the latter precedes formation of sucrose. The mechanism of sucrose synthesis is still uncertain. Some of the available evidence may be interpreted as indicating that it proceeds by a path different from that leading to glucose. By using an entirely chemical degradation method, they have proven that the hexoses are composed of two equal halves. They report that the trioses, hexoses, and glyceric acid are largely unphosphorylated. It appears that this may be due to the presence of a highly active phosphatase in the soybean leaves, the method of killing giving this enzyme an opportunity to operate.

In our first studies of photosynthesis with C^{14} (31), it was shown that cell constituents other than those which are extractable with hot water are tagged with surprising rapidity. As these substances seem certain to be secondary products not directly concerned with photosynthesis, they have not been extensively studied. Clendenning (32) has investigated the nature of the benzene-soluble substances from *Scenedesmus* which are tagged during

40 sec. of photosynthesis. He has conclusively shown that the activity found in this fraction is not due to contamination by minute amounts of water-solubles. Over 80 per cent of this activity is in the form of fats as is shown by recovery of 61 per cent in high molecular-weight fatty acids and 20 per cent in nonvolatile, water-soluble substances after vigorous saponification. None of the activity is in chlorophyll and only traces are in the carotenoids. He has pointed out that during photosynthetic exposures to $C^{14}O_2$ of 30 to 60 sec. the total amount of activity fixed in the lipid fraction is equal to that fixed in sucrose, and suggests that this rapid fixation of tracer in diverse substances must temper the interpretation put upon the large number of compounds found in the water-soluble fraction after relatively short exposures.

DARK FIXATION FOLLOWING ILLUMINATION

Normal dark fixation seems to have very little in common with photosynthesis (31). Some workers report (33) a short-lived increase in uptake of carbon dioxide in the dark immediately following exposure to light. Using tracer, a markedly enhanced dark fixation has been observed following a preceding period of photosynthesis (14) or a preceding illumination in the absence of carbon dioxide and oxygen [Benson *et al.* (34)]. In both cases the enhanced dark fixation shows characteristics of photosynthetic fixation and, indeed, it appears that we are here dealing with some of the first steps in the photosynthetic process. These dark "pick-up" reactions are, therefore, of considerable theoretical importance and have been studied systematically in the writers' laboratory [(14); Gaffron *et al.* (24)]. Fig. 1 is a schematic representation of the four principal ways in which labeled carbon has been used to detect dark fixation following illumination. These are: (I) label added during normal photosynthesis and cells killed the moment the light is turned off or at different times afterwards in the dark; (II) normal photosynthesis, label added the instant the light is turned off; (III) pre-illumination in the absence of oxygen and carbon dioxide until only a trace of photosynthesis persists, tracer carbon dioxide added as in (II); and (IV) same pre-treatment as (III), tracer carbon dioxide added in the light as in (I). The shaded parts after zero time (turning off of the light) represent the amount fixed in the dark; shaded parts before zero time represent photosynthetic fixation. The numbers refer to the percentage of total tracer fixed² which is found in phosphoglyceric acid and pyruvic acid. Scheme (III) comes nearest to the description of pre-illumination experiments reported by Benson *et al.* (34). As the results and conclusions from the two laboratories differ considerably, a rather detailed comparison seems in order.

Dark fixation following photosynthesis.—We have reported (14) that algae,

² In this case, and all others from our laboratory reported later in this review, only the water-extractable material is considered. In short-term experiments, this represents 95–100 per cent of all tracer fixed. The methods used for preparation of samples for counting and for determination of phosphoglyceric acid and pyruvic acid are given in references (14) and (15).

Scenedesmus, which have fixed tracer during photosynthesis fix an additional amount equivalent to about 10 sec. of photosynthetic fixation if they are allowed to stand in the dark a minimum of 20 sec. before killing (cf. Fig. 1, I). This is more than 20 times normal dark fixation in the period allowed. It must be emphasized that carbon dioxide is present in excess during both the photosynthetic and the dark periods. The magnitude of this dark fixation

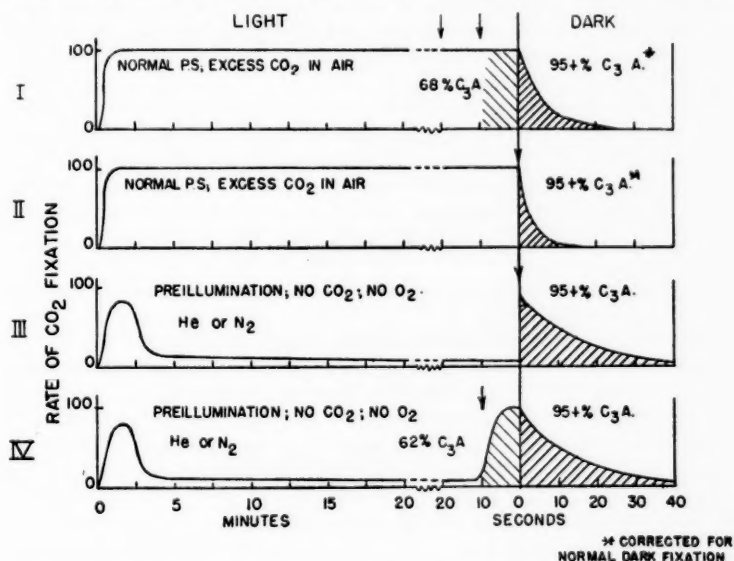


FIG. 1. Dark fixation of carbon dioxide following illumination. (*Scenedesmus*). Tracer carbon dioxide added at times marked \downarrow . Algae killed either the moment the light is turned off or at various times afterwards in the dark. Shaded parts before zero time represent photosynthetic fixation, shaded parts after zero time represent amount fixed in the dark. C_3A = Phosphoglyceric plus pyruvic acids.

is directly proportional to the photosynthetic rate (light intensity), always being equivalent to about 10 sec. of photosynthetic fixation with the light intensity employed. This indicates that it is intimately associated with the process of photosynthesis. It is not due to some unknown peculiarities of the first few seconds of tracer fixation, for the amount of fixation in the dark after photosynthesis at any one light intensity is independent of the time of preceding photosynthetic fixation (10 sec. or more). That it is a general property of normally photosynthesizing algae is shown by the fact that the dark fixation found after 4 hr. of photosynthesis with 4 per cent carbon dioxide in air is identical with that after 15 min. photosynthesis under the same conditions (Fager, unpublished observation). The half-life of the dark fixation reaction is 3 to 4 sec. at 20°C. This does not change when

the concentration of the substance(s) responsible for fixation is reduced by a factor of 5 by decreasing the light intensity [Gaffron *et al.* (24)].

Over 90 per cent of the tracer fixed² in 20 sec. in the dark by algae which have been photosynthesizing in 4 per cent carbon dioxide in air and then have fixed tracer for 10 sec. in the light (Fig. 1, I) is found in the carboxyl groups of phosphoglyceric acid and pyruvic acid (Fager, unpublished observation). If algae are allowed to photosynthesize with $C^{14}O_2$ and the tracer is added at the instant the light is turned off (Fig. 1, II), the dark fixation amounts to 25 to 35 per cent of that obtained if tracer is present for as little as 10 sec. before the light is turned off (14). Under these conditions, all³ tracer fixed (after normal dark fixation has been subtracted) is in the carboxyl groups of phosphoglyceric acid and pyruvic acid. When algae which have been illuminated in the absence of carbon dioxide and oxygen are allowed to fix tracer for 10 sec. in the light and then for 60 sec. anaerobically in the dark (Fig. 1, IV), the fixation in the dark is equal in magnitude to that in the light and all of it appears in the carboxyl groups of phosphoglyceric acid and pyruvic acid.

Dark fixation following illumination in the absence of carbon dioxide and oxygen.—Calvin & Benson (13) first showed that illumination of algae in the absence of carbon dioxide and oxygen results in an enhanced dark fixation if tracer carbon dioxide is added soon after the light is turned off. They suggested that this fixation represents the first steps in photosynthetic fixation and presented as proof the similarity of compounds tagged in the dark after such pre-treatment to those tagged after short periods of photosynthesis. However, their data (7), which is for *Chlorella*, shows markedly less tracer in phosphate esters after the dark fixation (38 per cent) than is present in these compounds after photosynthetic fixation (89 per cent).

We have performed similar experiments with *Scenedesmus* and find that, after 15 min. illumination in helium, the enhanced dark fixation occurs exclusively in the carboxyl groups of phosphoglyceric acid and pyruvic acid (Fig. 1, III), if estimated normal dark fixation under such conditions is subtracted (14). The correspondence between this result and the demonstrated importance of phosphoglyceric acid as the first stable compound in photosynthesis indicates that dark fixation after such pre-treatment does represent the carboxylation step in photosynthesis.

The amount of tracer carbon dioxide fixed by *Scenedesmus* in the dark after 15 min. illumination in the absence of carbon dioxide and oxygen is equivalent to 10 to 20 sec. of photosynthesis at 500 foot candles by an algal suspension of the same density (Fager, Gaffron & Rosenberg, unpublished observation). The dark fixation reaction has a half-life of 10 to 15 sec. at 20°C. The decay of the enhanced dark fixing power in the dark under helium has a half-time of about one minute at 20°C. The curves for fixation in phosphoglyceric acid alone and in phosphoglyceric acid plus pyruvic acid as a function of time follow exactly the curve for total fixation [Gaffron

³ In this case, and all others from our laboratory reported later in this review, qualify by "within experimental error (± 5 per cent)."

et al. (24)], showing that the only reaction occurring is the carboxylation leading to phosphoglyceric acid and pyruvic acid.

Effect of poisons.—It has been known for many years that the reactions connected with the carbon dioxide fixation and those leading to oxygen evolution differ very characteristically in their sensitivity to specific poisons (1). The former are mainly inhibited by cyanide; the latter by hydroxylamine, phthiocol, phenanthroline, etc. [cf. (35, 36)]. New experiments with chloroplasts have corroborated this inasmuch as not cyanide but hydroxylamine and *o*-phenanthroline act as specific poisons for Hill reactions [Madowall (37); Arnon & Whatley (38)]. In a preceding section we have discussed the evidence supporting the view that the carboxylation leading to phosphoglyceric acid is the reaction by which carbon dioxide enters the living cell in the course of photosynthesis. If this is true, the formation of phosphoglyceric acid should be inhibited by cyanide but not by hydroxylamine. We have shown that this is indeed the case [Gaffron *et al.* (24)].

The fixation of tracer carbon during photosynthesis is influenced by hydroxylamine in the same manner as by a decrease in the light intensity, and the fixation in the dark after pre-illumination (Fig. 1, III) is not influenced at all. To make experiments of the latter kind convincing is somewhat difficult because hydroxylamine penetrates much more slowly into the cells than cyanide, and the dark pick-up could run its course before significant amounts of hydroxylamine had entered the cell. For that reason, an unusually high concentration of hydroxylamine ($3.3 \times 10^{-3} M$) was used and added together with the tracer. Such high concentrations are, of course, unnecessary in ordinary photosynthesis experiments because $5 \times 10^{-4} M$ is sufficient to abolish photosynthesis in most algae and higher concentrations of the poison may give misleading results on account of hydroxamic acid formation (39) or the slow reduction of hydroxylamine itself.

Fortunately hydrocyanic acid enters the cell very quickly. This makes it possible to demonstrate its poisoning effect not only during the course of photosynthesis but also during dark fixation following illumination. The addition of cyanide ($10^{-3} M$) with the tracer carbon dioxide after previous illumination in the absence of carbon dioxide and oxygen (Fig. 1, III) produces a 70 per cent inhibition of the dark "pick-up" by *Scenedesmus*. Results such as these not only confirm the earlier kinetic deductions but point out the difference between the formation of phosphoglyceric acid and the carbon dioxide fixation in better known reactions, for instance, the formation of oxaloacetic acid or malate from pyruvate. Westheimer & Steinberger (40) have recently studied a decarboxylation which is catalyzed *in vitro* by heavy metal ions and would therefore presumably be sensitive to cyanide poisoning. In looking for similar cyanide-sensitive biological reactions, we find as useful analogies only the synthesis of formic acid from carbon dioxide and hydrogen in *Bacillus coli* (41), and the presumed intermediate decarboxylation of pyruvate during the fermentation of *Clostridium butylicum* [Kempner (42)].

The latter reaction is also sensitive to poisoning by carbon monoxide. This and the fact that photochemical reactions in chloroplasts are not

affected by carbon monoxide (43) require a reinterpretation of older experiments showing an inhibitory effect of carbon monoxide on photosynthesis in *Chlorella*, which was thought to be due to an inhibition of the oxygen evolution (44). That such a specific inhibition of photosynthesis by carbon monoxide really exists seems to be assured by recent experiments of Frenkel (45).

Significance of enhanced dark fixations.—From the foregoing experiments we learn that enhanced dark fixation occurs after illumination of algae and that the carboxyl groups of phosphoglyceric acid and pyruvic acid may account for all of the fixation within experimental error. In *Scenedesmus* this is the case; in *Chlorella* tracer is more widely distributed. Benson & Calvin (7) consider the similarity in the nature and distribution of compounds tagged during dark fixation following illumination in the absence of carbon dioxide and oxygen to that found after short periods of photosynthesis as proof that all reduction steps in photosynthesis occur in the dark. On this basis, they have postulated the formation of a "reducing power . . . in the form of a definite chemical species . . . supplied by the photochemical reaction involving the photolysis of water."

If one assumes that the process of photosynthesis is essentially the same in these two organisms, *Scenedesmus* and *Chlorella*, and there is no reason to believe that it is not, it is difficult to explain the apparent absence of a long-lived general reducing power (dark fixation does not progress beyond phosphoglyceric acid and pyruvic acid) in one and its presence in the other. Furthermore, as "sugar" is the probable product of photosynthesis, it is hard to understand why in *Chlorella* the reduction leads to nearly six times as much alanine (39 per cent) as sucrose (6.7 per cent). Even if the "phosphate esters" fraction (38 per cent) is entirely hexose phosphates, the reduction leading to alanine seems disproportionately large (7). We would interpret the results with *Chlorella* as due to its known greater dark metabolism which would tend to distribute tracer originally fixed in phosphoglyceric acid and pyruvic acid into other compounds. This explanation appears likely, for a major portion of the activity is in alanine, aspartic acid, and malic acid, all easily derived from pyruvic acid and thus from phosphoglyceric acid. The rate of such distribution would be greatly increased by the chance admission of even small amounts of oxygen during the dark period.

In trying to interpret the results obtained with *Scenedesmus*, the conclusion seems to us inescapable that the dark fixation proper involves one step only, the formation of phosphoglyceric acid. Some thermo-labile, but otherwise relatively long-lived, compound survives which causes this carboxylation. If it is a general "reducing power," such as triphosphopyridine nucleotide whose catalytic activity has recently been shown to be capable of coupling with the photochemical activity of illuminated chloroplasts (see p. 111), one may ask why the dark fixation is restricted to this single carboxylation. This is not the place to argue these questions in detail, but as we (14) have pointed out the simplest explanation for all the enhanced dark fixations following illumination so far observed is to assume that the

hypothetical 2-carbon acceptor for carbon dioxide or its dissociable addition product, the immediate "precursor" to phosphoglyceric acid, survive. In the case in which no carbon dioxide or oxygen is present during illumination (Fig. 1, III), it is the acceptor which survives in the dark; after normal photosynthesis (Fig. 1, I and II) it may be the acceptor or the precursor. If it is the latter, tracer added at the instant the light was turned off could be fixed only by exchange, and this might explain the reduction in the amount of tracer fixed in the dark under these conditions. Such a hypothesis would seem to conflict with the older observations (33) that showed a net uptake of carbon dioxide by plant material in the dark immediately following photosynthesis. However, all of these were noted under conditions where carbon dioxide might be expected to be limiting—high light intensity, low carbon dioxide pressure or cyanide poisoning—whereas this "pick-up" occurs at 500 foot candles with 4 per cent carbon dioxide in air.

Chemiluminescence.—Another type of after-effect has recently been discovered by Strehler & Arnold (46). This is a chemiluminescence of chlorophyll in living green plants and active chloroplasts following a preceding illumination of the plant material. The effect lasts for 30 sec. at normal temperature and up to 2 min. at the temperature of liquid nitrogen. The intensity of the chemiluminescence is a million times smaller than the light which initiates it but it can be recorded by a photomultiplier tube or on a photographic plate. There seems to be no doubt that chemical reactions involving newly-made products or intermediates of photosynthesis are responsible for an excitation of the chlorophyll molecule. Though the yields are extremely small, the process is probably similar to that occurring in the well known chemiluminescence of fireflies and luminescent bacteria. The poisons that are more likely to inhibit the reduction of carbon dioxide, cyanide and azide, enhance the chemiluminescence and those poisons which we know interfere with the evolution of oxygen, namely, hydroxylamine, phenanthroline, etc., inhibit it strongly. Since typical induction effects have been found after dark periods of several minutes, there is a good prospect that this chemiluminescence may turn out to be as valuable an indicator for certain reactions in the photochemical system as fluorescence has been (cf. Franck, this volume).

FORMATION OF THE HYPOTHETICAL C₂ CARBON DIOXIDE ACCEPTOR

The problem in the center of present interest is the source, nature, and mode of reaction of the hypothetical 2-carbon compound or fragment which reacts with carbon dioxide to give phosphoglyceric acid. Two facts are known: First, essentially all of the tracer found in phosphoglyceric acid and pyruvic acid after short periods of photosynthesis and after dark fixation following illumination is in the carboxyl groups (9, 14). This bespeaks the addition of carbon dioxide to a 2-carbon acceptor whether it is assumed that the acceptor is sufficiently reduced so that carbon dioxide addition directly forms phosphoglyceric acid or that a reduction follows the addition. The slowness of tagging of the α - and β -carbons of phosphoglyceric acid compared

to that of the carboxyl and the fact that no tracer, or only insignificant amounts, is found in likely 1-carbon compounds (9) speak against a 1-carbon unit mechanism. Second, tracer begins to appear in the α - and β -carbons of phosphoglyceric acid after relatively short periods of photosynthesis and this compound becomes uniformly labeled long before the entire cell or even the water-solubles (1 per cent of cell weight, 80 to 90 per cent of all tracer fixed up to 1 min.) reach this condition (9, 14). This indicates that the 2-carbon acceptor comes from part of the phosphoglyceric acid, i.e., that a relatively short and rapid cycle operates for the formation of this acceptor in amounts and at the rate necessary for photosynthesis. Under normal conditions, tracer appears in the β -carbon of phosphoglyceric acid as rapidly as in the α -carbon. This requires that the 2-carbon acceptor, or a precursor of this compound, shall be symmetrical.

Originally succinic, fumaric, tartaric, malic, and other acids were suggested as intermediates in photosynthesis because of their appearance as labeled compounds on radio-chromatograms of extracts from plant material which had fixed tracer photosynthetically for relatively short periods. Several cycles were subsequently proposed in which these 4-carbon compounds appear (34, 47). However, as stated in last year's review (2), these particular compounds have been eliminated as possible intermediates and thus the attractiveness of the originally proposed cycles on the basis of comparative biochemistry has disappeared.

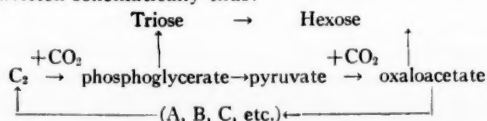
All of the cycles proposed by Calvin *et al.* have included two carboxylations. However, the hypothesis of two carboxylations requires at least two *ad hoc* assumptions in order to make it fit the observations. These assumptions are as follows: There must be at least four different reduction steps as against the one or two for which we have some evidence; all 4-carbon compounds must be thermo-labile so that they are destroyed during the usual extraction with hot water or alcohol.

Against the assumption that all 4-carbon compounds are thermolabile must be set the report of Aronoff & Vernon (17) that extracts from soybean leaves which had fixed tracer photosynthetically for short periods showed no great difference in the type of substances tagged whether killing with 80 per cent ethanol was done at -78°C . or at the boiling point.

If it is assumed that the cycle involves only one carboxylation, the only additional assumption necessary is a direct transformation of sugars into 2-carbon compounds (14). There are reports of the reverse reaction so that this one is not beyond the bounds of possibility.

We shall discuss in some detail the consequences of the most recently proposed cycles.

Two-carboxylation cycle.—The latest cycle proposed by Benson & Calvin (2) may be written schematically thus:



They have presented (9) as evidence for this cycle the fact that after short periods of photosynthesis (5 sec., 10,000 foot candles) phosphoglyceric acid (87 per cent), pyruvic acid (10 per cent), and malic acid (3 per cent) account for 100 per cent, within experimental error, of all tracer fixed. The small amounts of tracer found in malic acid were presumed to be derived from oxaloacetic acid by a side-reaction. Doubt is cast upon the involvement of the latter compound by the report of Aronoff & Vernon (21) that no tagged asparagine or aspartic acid is formed during 15 sec. photosynthetic tracer fixation by soybean leaves. As considerable amounts of tagged alanine are formed, presumably by reductive amination of pyruvic, it would seem that tagged aspartic acid should also be formed from oxaloacetic acid if the latter is present.

The arguments (9, 26) supporting this cycle on the basis of results of photosynthesis at very low light intensities (45 foot candles) are not convincing because under these conditions the respiratory fixation of tracer and the metabolic distribution of photosynthetically fixed tracer approach in importance the actual photosynthetic fixation. The early appearance of tracer in malic acid during the first few minutes under these conditions may be an entirely secondary process unrelated to photosynthesis.

It is difficult to write a scheme in which some of the compounds, A, B, C, etc., are not thermally stable and, therefore, determinable by the methods used. In the extreme case in which all of these compounds are assumed stable, one should find as much activity in other substances as is found in the carboxyl group of phosphoglyceric acid. With algae this has so far never been observed in photosynthetic periods of 10 to 15 sec. or less.

To get around these difficulties one might argue that all of the steps following a second carboxylation go extremely fast so that the carboxylation product of the 2-carbon acceptor, phosphoglyceric acid, is the only observable product. However, this possibility does not seem likely because the labeling of the 2-carbon fragment (α - and β -carbons of phosphoglyceric acid) is, from this point of view, a rather slow process. Tracer should appear in the α - and β -carbons of phosphoglyceric acid in much larger amounts than is found after very short periods of photosynthesis in algae [5 per cent after five sec. photosynthesis at 10,000 foot candles (9); 0 ± 2 per cent after 10 sec. photosynthesis at 500 foot candles (14)].

This difficulty may be met by assuming that the reservoirs of thermolabile compounds are very large and the reservoirs of stable compounds are very small. Tracer reaching the α - and β -carbons of phosphoglyceric acid in short periods of photosynthesis would thus be greatly diluted and most of the tracer in the cycle would be lost by thermal decarboxylations. However, the evidence of Aronoff & Vernon (17) in regard to cold killing indicates that very little, if any, tracer is fixed in thermally unstable compounds.

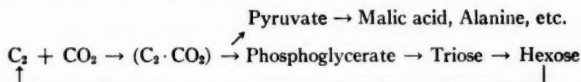
It might be argued that the small amounts of tracer found in the α - and β -positions of phosphoglyceric acid after short periods of photosynthesis are the result of non-photosynthetic side-reactions. Certainly cells which are actively photosynthesizing and growing have many possible entry ports for

infiltration of carbon dioxide by reactions entirely unrelated to photosynthesis, and it is surprising that tracer is initially as restricted in distribution as it is. On this premise, the cycle presented can be defended by assuming that the carboxylation of pyruvic acid does not have to proceed any faster than one-half the rate of carboxylation of the two-carbon acceptor (two moles of acceptor come from each mole of oxaloacetic acid) and that, therefore, it need only have completed the first step and all of this is lost during the killing. However, see the Aronoff & Vernon report in the paragraphs above.

The strongest argument against the two-carboxylation cycle is presented by the results of dark fixation immediately following photosynthesis in *Scenedesmus*. In this case there can hardly be any question of rates, for the algae are allowed to stand in the dark for 20 sec. If, as Benson & Calvin (7) suggest, all reductions are dark reactions by a general reducing power, then tracer should be fixed in oxaloacetic acid and thence by reduction in A, B, C, etc., and the α - and β -carbons of phosphoglyceric acid. However, all of the tracer fixed appears in the carboxyl groups of phosphoglyceric acid and pyruvic acid (72). To explain this observation, one must make the unlikely assumptions discussed above; that all compounds between pyruvic acid and the 2-carbon acceptor lose carbon dioxide when heated, and that the cycle functions very slowly. The latter is impossible, for the cycle must maintain a rate sufficient for photosynthesis, and turning off the light can have little immediate effect on its rate if all reactions in it are dark reactions. It appears that one must either discard the two-carboxylation cycle or introduce photochemical reductions into it, thus sacrificing the concept of a general reducing power.

Ochoa (28) has proposed a variant of the above cycle which, however, includes malic acid. The evidence presented earlier in regard to this compound makes its participation in the photosynthetic process doubtful.

One-carboxylation cycle.—We have proposed a cycle for the formation of the 2-carbon acceptor which involves only one carbon dioxide fixation (14, 24). Schematically it may be written thus:



The first objection to such a cycle is probably the unusualness of the reaction forming three 2-carbon acceptor molecules from one hexose. The same objection might be leveled at the two-carboxylation cycle, for all "expected" intermediates seem to have been ruled out and reactions and compounds which are unusual in biochemistry must be postulated.

According to the one-carboxylation cycle above, after very short photosynthetic contact with tracer only the carboxylation of the 2-carbon acceptor will have taken place and some of the tracer will have progressed through the pool of labile precursor to form phosphoglyceric acid. This will give rise to small amounts of pyruvic acid and perhaps traces of malic acid. The

small amounts of tracer reported to be in the α - and β -position of phosphoglyceric acid after short photosynthesis at high light intensities can be assumed to be the result of side-reactions by the actively metabolizing cells. The importance of malic acid would rise with decreasing light intensity which would decrease the rate of photochemical reduction of phosphoglyceric acid and thus proportionately favor the formation of malic acid. Tracer fixed in the dark immediately after normal photosynthesis or after illumination in the absence of carbon dioxide and oxygen will all be found in the carboxyl groups of phosphoglyceric acid and pyruvic acid unless metabolic reactions unrelated to photosynthesis have distributed it more widely. All of the above agrees with experimental results.

If tracer is added in the light, whether during photosynthesis or following illumination in the absence of carbon dioxide and oxygen, phosphoglyceric acid will be formed and a portion of it will be photochemically reduced. The proportion of tracer transformed into compounds beyond phosphoglyceric acid will depend only upon the light intensity and will be independent of previous treatment [in see Fig. 3, B and E in (14)].

Although some of the reductions in photosynthesis may be dark reactions connected with the photochemical apparatus by a considerable series of intermediates, we feel that photosynthesis involves at least one relatively direct photochemical reduction (cf. Franck, this volume, for physical evidence). Support for this concept is the observation by Gibbs (20) that the sucrose formed during 16 hr. tracer fixation in the dark by sunflower leaves has over 90 per cent of the tracer in the 3,4 position, whereas 1.5 min. fixation in the light results in 48 per cent in the 3,4 and 52 per cent in the 2,5 and 1,6 positions combined. This seems clear evidence that light is necessary to initiate the photosynthetic cycle forming the 2-carbon acceptor for carbon dioxide. Although sucrose synthesis may be the same in the light and in the dark from triose on, the steps preceding this compound in the light are very probably different from those in the dark.

It must be emphasized that none of the cycles so far proposed rests upon incontrovertible grounds. All schemes involve special assumptions in order to explain all experimental observations. Perhaps one of the most serious defects is, as Wood (23) has pointed out, the lack of information on specific activities. These are absolutely necessary before one can say with any certainty that compound A comes before compound B, etc. Ochoa (28) has stated the case clearly: "The results . . . still leave the detailed path of carbon in photosynthesis open to conjecture and speculation." This is not surprising in view of Wood's recent report (24) that the "seemingly straightforward reaction between oxalacetate, carbon dioxide and pyruvate" is not at all simple and its mechanism is still in doubt.

PHOTOREDUCTION

Some algae have the ability to adapt themselves, in an atmosphere of hydrogen, to an anaerobic metabolism resembling that of the purple bacteria. When illuminated, they evolve no oxygen but take up carbon dioxide

with an equivalent of hydrogen and, in the absence of carbon dioxide, even evolve free hydrogen (35). In view of the rapidity with which respiratory reactions may transform photosynthetic intermediates, the prospect of carbon dioxide reduction under anaerobic conditions appears as an attractive method of simplification. However, a major difficulty in using algae adapted to photoreduction lies in the need for low light intensities, i.e., low over-all reaction rates, in order to avoid any reversion to aerobic conditions. Such low rates may allow secondary fermentative transformations to catch up with the photosynthetic processes and the very purpose of the experiment is thus defeated. To circumvent this difficulty, the algae can be stabilized against reversion by poisoning with such inhibitors as phthiocol after adaptation (36). In this way, high rates of carbon dioxide reduction under anaerobic conditions can be enforced by using high light intensities. Our present knowledge concerning photoreduction indicates that it is, unfortunately, no simpler than photosynthesis.

Hydrogen evolution.—The ability to adapt to an anaerobic metabolism seems to be more widespread among algae and therefore of greater general significance than was originally assumed. To the three species of green algae, *Scenedesmus*, *Ankistrodesmus*, and *Raphidium*, in which it was found ten years ago, we may now add the green algae, *Chlamydomonas* and *Ulva lactuca* (48), the blue-green algae, *Synechococcus elongatus* (49) and *Chroococcus* sp., the brown alga, *Ascophyllum nodosum*, and the red algae, *Porphyridium cruentum* and *Porphyra umbilicalis* (48). Of these, *Chlamydomonas* is the most active. The rate of hydrogen evolution under the influence of light is 25 times as great in this alga as in *Scenedesmus* where it was originally discovered (cf. 35), and therefore reaches one-half of the rate recently reported by Gest, Kamen & Bregoff for the purple bacterium, *Rhodospirillum* sp. (50). The main difference between the algae and the purple bacteria in respect to this interesting reaction is, therefore, not so much the rate or the possible mechanism but its relation to the reaction by which elementary nitrogen is fixed. The fact that purple bacteria fix nitrogen was first suspected (50) when it was found that nitrogen gas inhibits the evolution of hydrogen in the light. It now appears that all photoreducing purple bacteria have the ability to fix elementary nitrogen. For *Chlamydomonas* and other green algae nitrogen is as inert as is helium. A correlation between the power to fix nitrogen possessed by some blue-green algae and the adaptability to hydrogen awaits further investigation.

Experiments with deuterium intended to determine the source of the hydrogen evolved in the light are difficult to interpret because of the possibility of last moment exchange on the hydrogenase surface. Frenkel's (48) results, in which hydrogen and not the deuterium present in the water was the main component of the gas evolved, seem to indicate that the hydrogen is released from organic substances which in the course of 2½ hr. are not equilibrated with the deuterium oxide in the suspension medium. In contrast to the hydrogenase of *B. coli*, the hydrogenase in *Chlamydomonas* does not quickly equilibrate deuterium and hydrogen between the aqueous and gas

phases and light has no influence upon the slow equilibration that does occur.

Carbon dioxide reduction.—We have done preliminary experiments on tracer carbon dioxide fixation by photoreduction in phthiocol-poisoned adapted algae [Gaffron *et al.* (24)]. Under these conditions, the transfer of labeled carbon into the fats and proteins was only slightly retarded. This result is interesting in suggesting that the photosynthetic mechanism in green plants radiates reactive intermediates in several directions and can speed up various syntheses without the aid of respiratory processes. We might have expected this outcome in view of the metabolism of the purple bacteria in which photoreduction is intimately associated with growth under completely anaerobic conditions. It appears that the incorporation of carbon dioxide by these bacteria is even more rapid than in the case of algae [M. D. Kamen (personal communication)].

Badin & Calvin (26) have also investigated photoreduction and the oxygen-hydrogen reaction in the presence of tracer carbon dioxide, and have identified some of the compounds containing radioactivity. No compounds other than those previously found in the radio-chromatograms of photosynthesis were discovered. The light intensity was 45 as against 500 to 10,000 foot candles used for photosynthesis experiments, and the duration of the experiments was hours as against seconds. The fact that cells which had been kept under anaerobic conditions for many hours came in contact with air for a few minutes between the illumination period and the killing made it necessary to introduce an "extrapolation (which) is not rigorously justified. . . ." It appears doubtful to us that valid conclusions can be drawn from their results in the absence of a set of dark controls especially designed to establish the extent and interference of secondary side-reactions under the experimental conditions employed.

REACTIONS OF CHLOROPLASTS

Ever since Hill (51) separated the photochemical reaction of green cells from the reactions involved in the assimilation of carbon dioxide, it has been evident that observations on the activity of chloroplast preparations rather than on the reduction of carbon dioxide would be essential for the understanding of the photochemical part of photosynthesis. Although it has not yet been possible to obtain chloroplast preparations or leaf macerates which show an increased carbon assimilation in the light,⁴ such preparations do retain their power to decompose water photochemically. If the appropriate oxidants, "Hill reagents," are supplied, oxygen is evolved. Most of these Hill reagents lie in an unphysiological potential range, but within the last year

⁴ This statement is no longer correct. By using special conditions for the preparation and testing of cell-free whole leaf macerates of spinach, it has been possible to demonstrate a definite and reproducible enhancement of tracer carbon dioxide fixation by illumination. This light-induced fixation is increased several fold by the addition of various low molecular weight compounds, of which glyoxal is perhaps the most interesting. Work is in progress to determine the site of this tracer fixation and the compounds which will give the greatest increase [Fager (unpublished observation)].

three which are of physiological importance have been added to the list: methemoglobin [Hill (24)], cytochrome-*c* (52), and oxygen (53, 54). TPN (triphosphopyridine nucleotide) may be a fourth example, though observations on oxygen evolution and carbon dioxide fixation [L. J. Tolmach (personal communication)] and malate synthesis [W. Vishniak & S. Ochoa (personal communication)] catalyzed by this substance in the light must be interpreted cautiously as indicating perhaps no more than an artificial coupling between the reduction of an unknown natural Hill reagent and some known reduction reactions by means of a known hydrogen transport substance. Nevertheless, the discovery that biological reducing agents, such as TPN, can be reduced by illuminated chloroplasts, however remote this reduction may be from the actual photochemical process, brings us one step nearer the possibility of *in vitro* photosynthesis.

Although algae, such as *Chlorella* and *Scenedesmus*, are not suitable for the preparation of chloroplasts, they can be made into "cellular chloroplast preparations" by treatment with quinone. Study of such preparations has given valuable information on the relation between quantum yields, saturation rates, and induction phenomena of chloroplasts and of whole cells.

Preparation of chloroplast material.—Progress in experimentation with chloroplasts depends upon the ability to obtain active and stable preparations. A comprehensive investigation of the photochemical activity of chloroplast preparations from many species [Clendenning & Gorham (55)] has shown that it varies greatly with the species of plant used. Among the land plants tested, millet, flax, chard, and spinach seem to be the best sources of chloroplasts in terms of high photochemical activity and low dark metabolism. The authors also made an exhaustive study of the dark reactions of chloroplast preparations and of the various factors in the processes of preparation and storage which contribute to the photochemical activity and to the stability of the preparations. They report that the original activity can be retained for weeks or months if the chloroplasts are frozen and stored at -40°C . Although freeze-drying causes the loss of 50 per cent or more of the original activity, it is very important to know that dried chloroplasts will work at all when re-wetted.

Arnon & Whatley (38) have made a careful study of the factors influencing oxygen production by illuminated chloroplast fragments. In addition to the striking effect of chloride (cf. section on *Effect of inorganic ions*), they investigated the thermolability of the photolytic system, its sensitivity to illumination preceding the addition of Hill reagents, and the effect of temperature, poisons, and pH on the rates and yields of oxygen evolution.

A true solution of a green enzyme which will show photochemical activity has not been obtained, but colloidal dispersions of sub-chloroplast particles with a molecular weight of a few million have been reported [Milner *et al.* (56)]. These dispersions still show one-quarter to one-half the activity of the original chloroplasts based on chlorophyll concentration. A considerable increase in activity results if they are agglomerated by salt precipitation. Ordinarily such sub-chloroplast preparations lose their activity in the course of

several hours but they can be kept active for a longer time by the addition of 10 to 15 per cent of methanol and, with this addition, they can be stored at -5°C . for several days.

Spikes *et al.* (57) have used a rapid and very accurate method for following the reactions of chloroplasts: Potential measurements to determine the oxidized/reduced ratio of the Hill reagent. With this method they observed marked changes with time in redox potential during preparation of the chloroplast material and notable differences between plant species (cf. 55). Such information may make it possible to select the source of chloroplasts and to modify conditions so that photosynthetic fixation of carbon dioxide can be demonstrated in a cell-free system.

Effect of inorganic ions.—Arnon & Whatley (58) have shown that sugar-beets and chard grown chloride-free photosynthesize normally. Therefore, chloride is not a coenzyme of photosynthesis as was once suggested. However, chloroplasts separated from chloride-free plants showed only feeble photochemical activity (oxygen evolution) unless chloride was added. If they were exposed to light before chloride was added, they were permanently inactivated. The authors suggest that some unknown substance essential for oxygen evolution is rapidly inactivated by light but is protected by chloride. They have also reported (38) that the addition of potassium chloride (.01 *M*) to normal chloroplast preparations increases the rate of oxygen evolution, often by a factor of three. This increase in rate makes it possible to obtain stoichiometric yields of oxygen from the added oxidant before an appreciable thermal deterioration of the chloroplast material can occur [cf. Warburg & Lüttgens (43)].

Pirson & Wilhelmi (59) have continued earlier investigations on the photosynthesis of cells grown in manganese-free media. When *Ankistrodesmus* was grown at an intensity of 800 lux in the absence of manganese salts, no particular chlorosis developed, but the photosynthesis of such cells at light saturation was very much depressed (about one-quarter of normal). Upon addition of manganous ion (10^{-6} *M*), the rate of photosynthesis increased rapidly and after about 90 min. was equal to that of the normally grown control. Compared with a similar effect of potassium added to cells suffering from a lack of potassium, the manganese reaction is not only faster but much more pronounced.

Working with crude suspensions of *Avena* chloroplasts, Gerretsen (60) has observed that the addition of catalytic amounts of manganous ion results in an increased oxygen uptake and a marked increase in redox potential during illumination. These effects are absent in the dark. He interprets the increase in redox potential as an indication of the formation of hydrogen peroxide by photochemical reduction of oxygen. Such an interpretation seems likely, for Mehler, in this laboratory, has shown (unpublished observation) that manganous ion has a strong catalytic effect on oxygen uptake by a system in which hydrogen peroxide is known to be formed in this manner (cf. section on *Oxygen as a Hill reagent*).

Spikes *et al.* (57) have found that illuminated chloroplasts can not hold

the redox potential of a saturated ferrocyanide solution to that for ferrous ion. They have calculated the amount of oxidant necessary to produce such a result and conclude that it must be due to an enzymatic oxidation of ferrous ion by molecular oxygen.

Enzymes.—Waygood & Clendenning (61) have made a survey of the carbonic anhydrase content of plant material. The highest activity was found in the cytoplasm of spinach, New Zealand spinach, and elderberry. The activity in albino plants was somewhat below that in green plants of the same species. The most striking result was the observation that the carbonic anhydrase activity of aquatic plants is less than one-hundredth that of land plants. The authors suggest that this enzyme may be one of those which limit the overall rate of photosynthesis at saturation. However, the relatively low content of this enzyme in aquatic plants is not reflected in their photosynthetic rates, which are as high or higher than those of land plants.

Eyster (62) has shown that catalase activity parallels chlorophyll content in corn.⁵ When the plants are grown in the light, albino corn has about one-seventh the catalase activity of normal green corn. When grown in the dark, green and albino plants have equal activity, both slightly above that of green corn grown in the light. Exposure of dark-grown plants to light results in a rapid change in catalase contents to those usual in light-grown plants. In view of the demonstration that hydrogen peroxide is not the photoperoxide produced by photolysis of water (cf. next section), the participation of catalase in photosynthesis is doubtful.

Arnon (63) has shown that the copper-protein enzyme, polyphenoloxidase, is intimately associated with the chloroplast fraction of the extract obtained from macerated leaves of chard. This localization is at variance with that found for spinach and sugar-beets by earlier investigators but the differences may be due to preparatory factors. Because of the reported absence of this enzyme in many common green plants, it seems doubtful that it can be closely related to photosynthesis.

Oxygen as a Hill reagent.—The action of oxygen as a Hill reagent is of particular interest because of its inhibitory effect on photosynthesis (cf. Franck, this volume). When spinach chloroplasts are mixed with an excess of commercial catalase and 2 per cent ethanol, oxygen is taken up continuously upon illumination, and an equivalent of acetaldehyde appears [Mehler & Gaffron (53)]. Although catalase is certainly present in spinach chloroplasts, they do not show the oxygen-alcohol reaction unless a considerable excess of catalase is added. An explanation for the need of so much enzyme can be found in Chance's observation that the catalatic decomposition of hydrogen peroxide is about a million times faster than the peroxidatic reaction with alcohol (64). Acetaldehyde is not formed in the dark, nor in the light if oxygen is absent. This latter observation proves that the photoperoxides resulting from the photolysis of water are not hydrogen peroxide since the

⁵ Exactly the reverse relationship between chlorophyll and catalase has been reported by several investigators.

Keilin-Hartree reaction is specific for very low concentrations of this substance. It follows that oxygen is reduced photochemically to hydrogen peroxide and is, therefore, a Hill reagent. If this hydrogen peroxide is not quickly removed by a reductant, the photochemical reaction may stop or it may continue in such a way that the hydrogen peroxide reacts with the photoperoxides and reforms free oxygen. If the latter is true, it should be possible, using oxygen isotopes, to demonstrate a continuous exchange of oxygen between water and the gas phase during illumination.

If quinone is added in the course of an experiment with oxygen, ethanol, and catalase, the absorption of oxygen changes immediately into oxygen evolution at precisely the rate expected for the undisturbed quinone reduction (52, 53, 54). After this preferred reaction has nearly reached completion, the absorption of oxygen recommences. Following such treatment, the peroxide-ethanol reaction proceeds at a rate nearly twice as great as before the addition of quinone. This catalytic effect of quinone can also be demonstrated when it is reduced in the dark in the presence of chloroplasts by an equivalent amount of ascorbic acid. When illuminated, this pre-treated material immediately shows the accelerated uptake of oxygen. Hydroquinone does not evidence an appreciable catalytic effect on oxygen uptake.

Ascorbic acid cannot replace ethanol as a reductant for the hydrogen peroxide formed during the illumination of chloroplasts in the presence of oxygen but an excess of this compound, in the presence of small amounts of quinone, increases the rate of oxygen absorption still further (Mehler, unpublished observation). The rate of oxygen consumption is now as great as the rate of photosynthesis would be in living cells containing the same amount of chlorophyll. The simplest explanation is that under these complicated conditions the Hill reaction proceeds without an evolution of molecular oxygen, the photoperoxides being used in the oxidation of alcohol and ascorbic acid. Proof of this hypothesis awaits the results of experiments with oxygen isotopes.

Chloroplasts and hydrogen-transport systems.—Tolmach (unpublished data) in this laboratory has obtained as high as twenty-fold increases in oxygen evolution from illuminated leaf macerates by the addition of catalytic amounts of TPN. He has also found that illumination of a washed-chloroplast preparation containing TPN, "malate enzyme," [cf. Wood, Ochoa (24); Conn, Vennesland & Kraemer (65)], pyruvate, and tracer carbon dioxide increases the tracer fixation by a factor of ten or more over dark fixation. Simultaneously, Vishniak & Ochoa (personal communication) have found that the synthesis of malate by chloroplasts in the same system ($C^{13}O_2$) is increased by illumination. They used a spectrophotometric method for determination of malate synthesis. They have also obtained an enhanced production of lactate upon illumination of a system composed of chloroplasts, diphosphopyridine nucleotide, lactic dehydrogenase, and pyruvate. The relationship of these observations to photosynthesis is unknown at present and their interpretation must be tempered by the fact that the reactions observed differ considerably from those which are indicated as occurring in

photosynthesis. However, the demonstration that an enzyme-substrate system known to perform reductions by thermochemical reactions can be coupled with the photochemical reactions of chloroplasts is of greatest importance.

This coupling of photochemical reactions with a known hydrogen-transport system might be taken as a model for the stored reducing power postulated by Benson & Calvin (7). (However, cf. section on *Significance of enhanced dark fixations*.) In this connection, Mehler's observation (52) that no evidence for a stored reducing power was obtained with spinach chloroplasts in experiments capable of detecting as little as 1 per cent of the calculated amount of this hypothetical reducing power indicates that the usual Hill reactions may not require an intermediate hydrogen transport system.

Cellular chloroplast preparations.—Quinone penetrates easily into living algal cells. Once inside the cells, the predominant reactions of this compound vary greatly with the plant species [Mehler (52, 53, 54)]. In some marine red algae, respiration increases under the influence of quinone, photosynthesis is eliminated, and light has no influence on the further trend of metabolic events. A species of blue-green alga, *Tolypothrix*, shows a diminished Hill reaction with quinone when illuminated. Small amounts of quinone abolish photosynthesis irreversibly and inhibit respiration very strongly, if not completely, in the green algae, *Scenedesmus*, *Chlorella*, and *Ulva*. The algae are killed and made into "cellular chloroplast preparations" by this treatment [Clendenning & Ehrmantraut (66, 67)]. Such preparations show a high rate of photochemical oxygen evolution in the presence of sufficient quinone but the yields are always below the 90 to 95 per cent of the theoretical which can easily be obtained with washed chloroplasts. Similar reduced yields are reported for chloroplasts tested in their own juice with quinone. Very high concentrations of quinone completely inhibit all photochemical reactions.

These "cellular chloroplast preparations" have proven very useful for establishing or confirming the following facts: The highest rate of oxygen evolution (saturation rate in strong light) (66) and the highest efficiency (quantum yield in low light) (67) are identical with, or only slightly less than, those for photosynthesis in the same algae. The saturation rate appears to be limited by a "Blackman period" of the same duration (0.01 sec. at 25°C.) as in photosynthesis. Consequently this dark reaction cannot be connected with the reduction of carbon dioxide. As studies on photoreduction (68) have shown that it is not involved in the evolution of molecular oxygen, it must be connected with the photochemical decomposition of water [Clendenning & Ehrmantraut (66)].

There is little indication that the quantum efficiency of chloroplast reactions can be brought up to that reported by Warburg & Burk (69) for dense, acid suspensions of *Chlorella*. The high efficiency of photosynthesis found by these authors seems to remain a unique phenomenon, for, in addition to the many measurements reported in previous years giving about 0.1 as the maximum quantum efficiency, we now have two new reports, Larsen (unpublished thesis), on green bacteria and Yocum & Blinks (70) on brown and red algae,

which confirm the value of 0.1 as a kind of natural constant. The last mentioned work is a continuation of the long-awaited paper of Haxo & Blinks (71) on the photosynthetic action spectra of marine algae.

The lag in reaching the final steady rate of photosynthesis after a preceding period of darkness (induction period) is absent in reactions with chloroplasts and "cellular chloroplast preparations" (66). Obviously this finding relegates the cause for the induction period to the reactions necessary to establish the cycle promoting the fixation and reduction of carbon dioxide (cf. Franck, this volume).

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BIOSYNTHESIS OF CHLOROPHYLL AND RELATED PIGMENTS

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GENERAL CONSIDERATIONS

This review is concerned primarily with the biochemical origin of chlorophyll and related tetrapyrrolic pigments. Since the synthesis of tetrapyrroles occurs in all living cells, and has been studied in microorganisms and animals as well as in plants, contributions to our knowledge from all these sources will be reviewed here.

The two major pigments of protoplasm are the red iron-containing pigment heme or iron-protoporphyrin and the green magnesium-containing pigment chlorophyll. That these two pigments were related in structure was first demonstrated by Hoppe-Seyler in 1880 (1), when he found that drastic treatment of chlorophyll with alkali converted the green pigment into red pigments having absorption spectra that resembled red pigments derived from iron protoporphyrin. Chemical studies of the structure of these compounds were actively pursued for the next fifty years. The work of Nencki, Piloty, Marchlewski, Küster & Willstätter (2) demonstrated that heme and chlorophyll were built upon the same basic tetrapyrrole plan. It remained for Fischer (3) and Conant and their coworkers to determine the composition and positions of side chains and to make clear the differences in structure between these two substances.

With the establishment of the structures of heme and chlorophyll it became apparent that a common pathway of biosynthesis for these molecules might exist. Advances of the last few years tend to support this idea of a common pathway and make possible the formulation of a scheme for chlorophyll biosynthesis. The scheme of chlorophyll and heme biosynthesis shown in Figure 1 is presented as a framework for organizing and discussing the experimental data and hypotheses of this review.

The biosynthetic chain of chlorophyll begins with the small building blocks, acetate and glycine molecules, which are part of the basic metabolic milieu. These small molecules are condensed in a series of n steps to form the complex molecule protoporphyrin (Fig. 1, Formula IV). From protoporphyrin two classes of compounds are formed, namely: the iron porphyrins or hemes; and the magnesium porphyrins which give rise eventually to chlorophyll. According to this scheme, heme and chlorophyll are related to each other biochemically since both arise from the same precursor molecule, protoporphyrin.

These two pigments are also related to each other functionally (106). Studies on photosynthesis, especially the work on isolated chloroplasts by

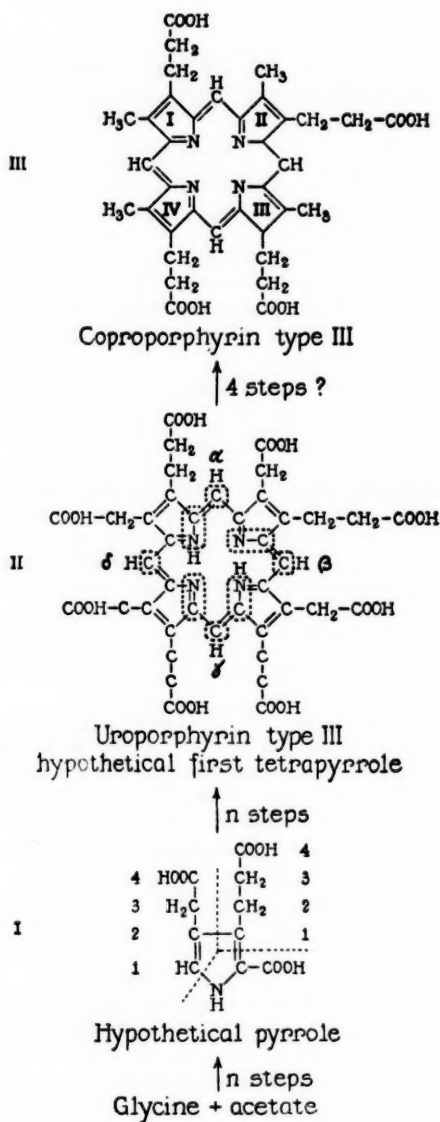


FIG. 1a. Scheme for heme and chlorophyll biosynthesis.

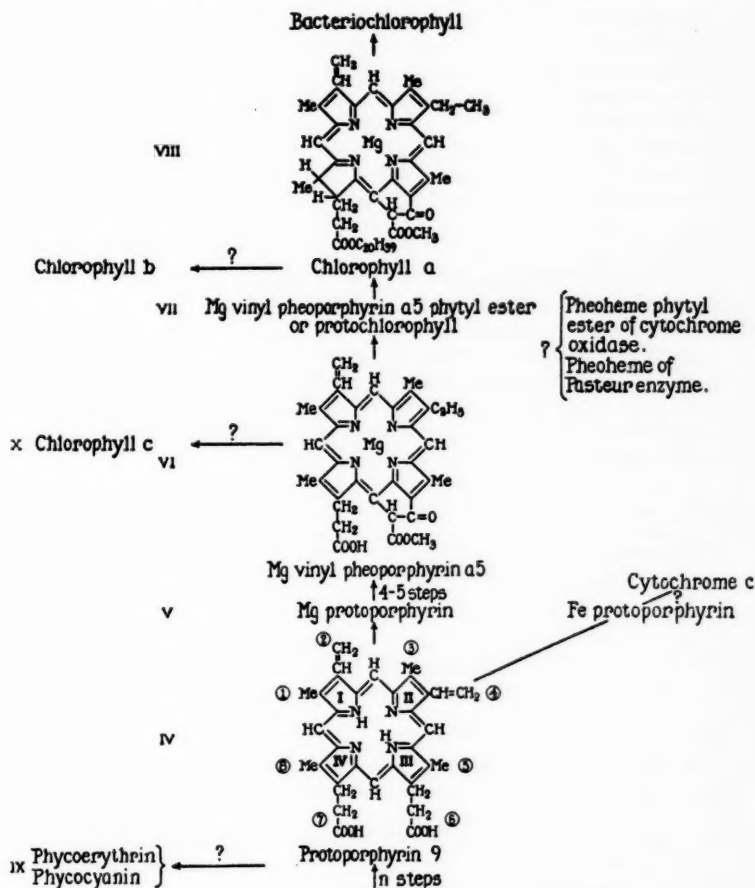
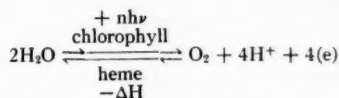


FIG. 1b. Scheme for heme and chlorophyll biosynthesis.

a number of investigators [reviewed by Holt & French (4)] have revealed the stoichiometric relations expressed by the forward reaction of the equation:



where (e) represents electrons of high potential energy, the energy of which

will be stored primarily in the configuration of organic substrates. In this reaction of photosynthesis the storage of the energy of sunlight is catalyzed by the porphyrin pigment, chlorophyll. If four quanta of light are required (i.e. $n=4$), as suggested by the experiments of Warburg & Burk (5), then the energy or reducing capacity of (e) would be in the neighborhood of the hydrogen potential. The reaction in the reverse direction represents oxidation with oxygen to form water, energy being thereby released. This oxidation is catalyzed by a series of heme enzymes containing an iron porphyrin as prosthetic group.

The formulation of the biosynthetic chain of chlorophyll rests on certain assumptions that have been the basis for constructing biosynthetic chains in general. We may consider them here very briefly.

(a) The first assumption considered as axiomatic is that a biological compound is synthesized or changed in small discrete steps converting a compound 1 \rightarrow 2 and compound 2 \rightarrow 3, etc. (Fig. 2). These postulated steps must be reasonable from a biochemical or organic chemical point of view. (If several pathways might be constructed, then additional evidence must be secured as to which pathway actually is present.)

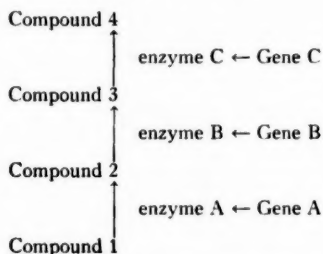


FIG. 2. Diagrammatic formulation of assumptions in construction of a biosynthetic chain.

(b) The second assumption is that each of these stepwise changes is brought about by a specific enzyme so that enzyme A acting on compound 1 converts it to compound 2, and enzyme B acting on compound 2 converts it to compound 3, etc.

(c) The third assumption is that a gene A gives rise directly or indirectly to enzyme A; gene B gives rise to enzyme B, etc. Opinions differ as to the wisdom of including the concept of the genes in considerations of the biosynthetic chain. However, the hypothesis of this gene-enzyme relationship has proven so fruitful from a methodological point of view in the study and analysis of biosynthetic chains that it is incorporated here.

It will be seen from Fig. 2 that complete identification of a biosynthetic chain would require knowledge of the substrates, enzymes, and genes concerned. The best example of a biosynthetic chain is the glycolytic scheme

where most of the intermediate compounds and enzymes are known in the reversible reactions of pyruvic acid to starch or glycogen. In most cases, however, only a few substrates scattered along various biosynthetic chains are known. In the work to be reviewed, the data on the biosynthetic chain of chlorophyll are fragmentary. At present the formulation of this biosynthetic chain rests mainly on the first assumption, namely, that biosynthesis proceeds in a stepwise manner and that a number of known compounds may be assembled into a scheme that is compatible with our knowledge of organic and biological chemistry.

BIOSYNTHESIS OF PROTOPORPHYRIN

The occurrence of large amounts of protoporphyrin in mutant *Chlorella* cells suggests that protoporphyrin is a precursor in chlorophyll biosynthesis. Within recent years, tracer techniques have been used to study the precursors of iron protoporphyrin, which is the prosthetic group of hemoglobin and of the heme enzymes, catalase, peroxidase and, cytochrome-*b*. These tracer studies, performed primarily on the red blood cell, have shown that certain small molecules are the main source of the nitrogen and carbon atoms of the protoporphyrin of the red blood cells. Since the steps in the synthesis of protoporphyrin are undoubtedly identical in both the plant and animal cells, it will be necessary to consider here, in connection with the steps in the biosynthesis of chlorophyll, the data derived from the tracer studies on red blood cells. A number of reviews of this rapidly developing subject of protoporphyrin synthesis have already appeared by Shemin (6), Bentley (7), Lemberg & Legge (8, 11), Maitland (9), and Rimington (10).

Source of the nitrogen of protoporphyrin is glycine-N.—In a now classical paper, Shemin & Rittenberg (12) demonstrated that glycine labeled with N^{15} , when fed to a human, is incorporated into the heme of newly formed red blood cells. Other compounds labeled with N^{15} , such as proline, glutamic acid, leucine, and ammonium citrate were utilized only slightly, i.e., only to the extent one might expect after feeding a nonspecific source of nitrogen that diluted the total body nitrogen with N^{15} . The incorporation of N^{15} from glycine into pyrrole nitrogen has now been demonstrated in man, rat, duck, *Corynebacterium diphtheriae* and *Serratia marcescens*. Shemin & Rittenberg (13) were also able to estimate the life span of the red blood cell in man using labeled glycine, since N^{15} was incorporated into the heme of newly formed red cells during the first days of ingesting labeled glycine and the red cells only lost their heavy nitrogen after they had degenerated—a matter of some 120 days. This experiment also provided the first example of a protein, hemoglobin, which is not in a constant process of breakdown and resynthesis.

In attempting by tracer techniques to follow the incorporation of small molecules which are normal constituents of a metabolic pool, the ideal plan would be to work with the specific enzyme systems which permit synthesis of the desired product. The use of tissues or cells instead of the whole body enhances the value of the experiment by limiting to some extent the enzyme

systems present. The discovery by Shemin, London & Rittenberg (14) that nucleated red blood cells of the duck would synthesize heme *in vitro* permits direct addition of labeled compounds *in vitro* to the cells which contain only a limited number of enzyme systems, as contrasted with the whole organism.

The synthesis of heme appears to take place only in immature red blood cells (i.e., reticulocytes). London, Shemin & Rittenberg (15) have demonstrated the synthesis of heme *in vitro* by normal rabbit reticulocytes. In addition to immaturity of the cells, other factors for normal hemoglobin formation are required. For example, in sickle cell anemia of humans the non-nucleated but immature red cells may or may not be capable of forming heme *in vitro*. The presence of a nucleus in the red cell is in itself no criterion for heme synthesis, since, although all circulating avian red cells are nucleated throughout their life span, it appears that heme synthesis is only accomplished by the immature red cells of the duck; these constitute about 20 per cent of the total red cells.

The N^{15} of labeled serine has also been found to be incorporated into heme with as high an efficiency as is labeled glycine. This is postulated to occur by the reversible conversion of serine to glycine on the following evidence: when isotopic serine was mixed with large amounts of nonisotopic glycine, the isotope concentration in the newly formed heme was very low. On the other hand, when isotopic glycine was incubated with large amounts of nonisotopic serine the labeled heme contained a high N^{15} content.

That glycine nitrogen was utilized equally for the formation of the two types of pyrrole rings in protoporphyrin was established by Wittenberg & Shemin (16) and independently by Muir & Neuberger (17). [In protoporphyrin (Fig. 1, formula IV), the pyrrole rings I and II have methyl and vinyl groups as side chains; pyrrole rings III and IV have methyl and propionic acid groups as side chains.] The determination of the labeling of N^{15} in these two types of pyrroles was accomplished by first converting the labeled heme to hematoporphyrin dimethyl ether with hydrogen bromide in acetic acid and subsequent esterification. The vinyl groups were thus converted to $-\text{CH}(\text{OCH}_3)-\text{CH}_3$. The resulting porphyrin was degraded by oxidation with chromic acid in sulfuric acid, producing a mixture of methyl methoxy-ethyl maleimide (XI) derived from pyrroles I and II; and hematinic acid (XII) derived from pyrroles III and IV. These two compounds were separated by extracting the hematinic acid from ether into aqueous bicarbonate. The two compounds were found to be equally labeled with N^{15} . Muir & Neuberger (17) describe the synthesis of these imides, their absorption spectra, and dissociation constants.

Only the α -C atoms of glycine are used in the synthesis of protoporphyrin.—Since the nitrogen of glycine is utilized for heme synthesis, it seemed probable that the carbon atoms of glycine might also be incorporated. Altman *et al.* (18) fed rats glycine labeled in the α -carbon atom with C^{14} and showed that this carbon atom was incorporated into heme. Grinstein, Kamen & Moore (19) showed that the carboxyl-carbon of glycine is not incorporated.

Wittenberg & Shemin (20) proved that the α -carbon atom derived from glycine occurs in the α -ring carbon of the pyrrole on the side of the vinyl or propionic acid groups rather than on the side of the methyl groups [Fig. 1

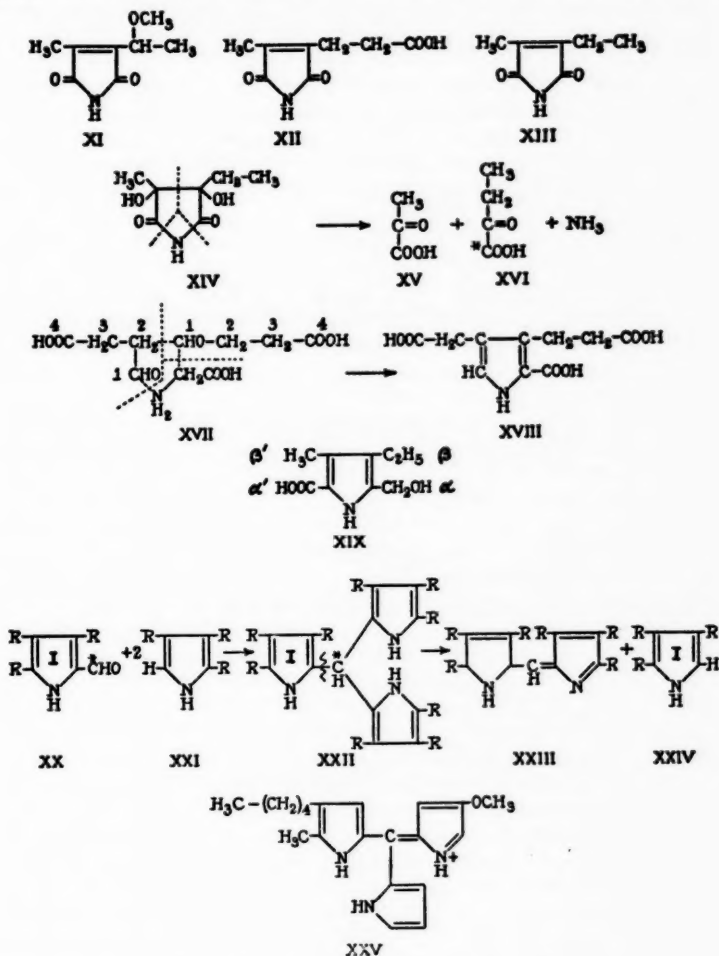


FIG. 3. Formulas of some pyrrole compounds.

(IV)]. When duck red blood cells were incubated with α -carbon¹⁴ labeled glycine, labeled heme was formed. This was converted to mesoporphyrin. This porphyrin was oxidized to methyl ethyl maleimide [Fig. 3 (XIII)]

(derived from pyrrole rings I and II of the porphyrin) and to hematinic acid [Fig. 3 (XII)], derived from pyrrole rings III and IV of the porphyrin. The hematinic acid was decarboxylated in a bomb tube, using ethanol plus ammonia to convert it to methyl ethyl maleimide. The methyl ethyl maleimides derived from each pair of pyrroles were then oxidized with NaClO_3 plus OsO_4 to 2-methyl-3-ethyl tartarimide (XIV) which was cleaved by sodium metaperiodate into ammonia, pyruvic acid (XV), and α -keto butyric acid (XVI). The acids were separated chromatographically. Only the α -keto-butyric acid was found to be radioactive; splitting off the carboxyl to carbon dioxide by oxidation, revealed that this carbon was the labeled atom, thus demonstrating that the α -carbon on the vinyl or propionic acid side of the pyrrole is the one derived from the α -carbon atom of glycine.

Radin, Rittenberg & Shemin (21) using duck red cells *in vitro* found that a total of 8 α -carbon atoms of glycine were incorporated into heme. The analysis of Wittenberg & Shemin (20) revealed that one α -carbon atom (the one attached to the nitrogen of glycine) was incorporated into each of the four pyrrole rings and the other four α -carbon atoms derived from four additional glycine molecules constituted the methene bridge atoms, α , β , γ , δ , joining the pyrrole rings together [Fig. 1 (II)].

The other carbon atoms of protoporphyrin appear to arise indirectly from acetate.—The first experiment indicating the incorporation of acetate into heme was carried out by Bloch & Rittenberg (22) in 1945, using acetate labeled with heavy hydrogen. More recently, Ponticorvo, Rittenberg & Bloch (23), using acetate labeled with deuterium in the methyl group, found that one-third of the hydrogen atoms of heme were derived from the methyl groups of acetate molecules. Using C^{14} labeled acetate, Radin, Rittenberg & Shemin (21) reported that both carbon atoms of acetate as well as the α -carbon atom of pyruvate were used, but not acetone or carbon dioxide. The carboxyl carbon atoms of heme were derived from the carboxyl carbon atoms of acetate. The methyl carbon side chains of the hemes around the ring [Fig. 1 (IV)] were derived from the methyl carbon of acetate as also were the carbon atoms of the pyrroles to which these methyls were attached. Muir & Neuberger (24) have studied the origin of the carbon atoms of heme and confirmed certain of the above data, finding also that at least one of the vinyl carbon atoms could be derived from the methyl carbon of acetate.

These investigations suggest that acetate may be the origin for most, if not all, of the carbon atoms, other than those from glycine, and also suggest that all of the pyrroles are derived from a common precursor. On the assumption of uroporphyrin [Fig. 1 (II)], as precursor of protoporphyrin, Lemberg & Legge (8) and Neuberger, Muir & Gray (25) have proposed the hypothesis that α -ketoglutarate is the more immediate precursor. The α -ketoglutarate would be derived from the condensation of acetate with oxaloacetate to form citrate, and the citrate would eventually be oxidized to α -ketoglutarate via the citric acid cycle.

Unpublished degradation studies by Shemin & Wittenberg on the proto-

porphyrin of red blood cells fed labeled acetate, have given the percentage of labeling in each carbon atom in the pyruvate and α -ketobutyrate molecules [Fig. 3 (XV and XVI)]. On the basis of the new data, these workers postulate as immediate precursor a 4-carbon compound, perhaps on the oxidative level of succinic semi-aldehyde (i.e., $\text{CHO}-\text{CH}_2-\text{CH}_2-\text{COOH}$). The production of the hypothetical pyrrole [Fig. 3 (XVIII)] would require the condensation of glycine plus two of these four carbon units (XVII); the carbon atoms having the same numbers would have the same percentage of activity of labeling [cf. Fig. 1 (I & II)].

The biological formation of tetrapyrrolic porphyrins by condensation of monopyrroles.—This subject can as yet be discussed only in terms of hypotheses (8, 26). It is known, however, that the methene bridge carbon atoms linking the pyrroles together are derived indirectly from the α -carbon atoms of glycine molecules. The possibility that the α -carbon of glycine gives rise to an active one-carbon unit on an oxidative level with that of formic or formaldehyde is suggested by various new data which have appeared within the last year but cannot be detailed here. Folic acid and vitamin B₁₂ seem to be intimately connected with the formation and utilization of this one-carbon unit in the formation of serine from glycine, methionine from homoserine, and the insertion of a one-carbon unit in the formation of purines. Whether the one-carbon unit is utilized directly or indirectly to link the pyrroles together is not known. Nor is there evidence to explain the method of formation of the ubiquitous type III porphyrin isomer which is unsymmetrical. Since the symmetrical type I porphyrins occur generally in minute amounts and are increased only in diseased states, it is possible that in such diseased states a lesion occurs in the limited ability to form the last and unsymmetrical condensations at the γ , δ -carbon positions [Fig. 1, (II)].

Formulas I and II (Fig. 1) summarize the data and hypotheses on the origin of the atoms of uroporphyrin from glycine and acetate. Eight glycine molecules and eight 4-carbon units (possibly succinic semi-aldehydes) would be required. The nitrogen-carbon atoms, ringed together, are both derived from the same glycine molecule. Each separately ringed carbon atom is derived from the α -carbon atom of another glycine molecule via a one-carbon unit. On the basis of the labeling of protoporphyrin, Wittenberg & Shemin (20) have suggested a numbering scheme for uroporphyrin type III.

The organic-chemical methods of porphyrin synthesis.—Fischer and co-workers (3, 35) have generally involved the synthesis of dipyrromethanes or dipyrromethenes and their condensation to the tetrapyrroles. A condensation of monopyrroles to porphyrins under mild conditions has been reported by Siedel & Winkler (27). For example, a pyrrole [Fig. 3 (XIX)] when dissolved in methanol and left exposed to air at room temperature, condenses spontaneously to form a mixture of type I and II etioporphyrins, i.e., porphyrins with methyl and ethyl side chains. It is interesting to note, on analogy with the naturally occurring porphyrins, that both substituents

in the β, β' -positions of the pyrrole must be present to obtain the porphyrins. Since the porphyrins can only form if the pyrroles are bound together in a *cis*-configuration, the possibility suggests itself that the presence of substituents in the β positions favor the *cis*-configuration. The problem of *cis* and *trans*-isomers in the open chain di- and tetra-pyrroles has as yet received scant attention.

The interesting method of pyrrol aldehyde condensation may have biological implications. The condensation of pyrrol aldehyde with α -unsubstituted pyrroles under mild conditions to form dipyrroles has been further elaborated so that porphyrins may be obtained in relatively high yields. It had previously been shown by Corwin & Andrews (28) that a pyrrol α -aldehyde [Fig. 3 (XX)] would condense with two α -unsubstituted pyrroles (XXI) to give rise to a tripyrrol-methane (XXII) as an intermediate, followed by a cleavage to form a monopyrrole (XXIV) plus a dipyrromethene (XXIII). Here the pyrrol aldehyde serves solely as the source of the methene C* joining the two pyrroles. In a continuation of this study Andrews, Corwin & Sharp (29) used pyrrol α -aldehyde to condense with 5,5'-unsubstituted dipyrrol methenes to form leuco porphyrins which oxidized spontaneously to a porphyrin in 40 per cent yield. The condensation took place in absolute ether saturated with dry HCl, over a period of ten days at room temperature. The intermediate steps could not be isolated. For the bridge source the α -aldehyde of N-methyl pyrrol α -aldehyde was used, but it was also found that the β -pyrrol aldehyde might be used; formic acid could also be used as the bridge carbon, but yields of the porphyrin were not as satisfactory.

This mechanism is biochemically interesting because a similar mechanism might be at work, at any rate in the formation of the red tripyrrol-methene pigment, prodigiosin [Fig. 3 (XXV)] produced by the common bacterium *S. marcescens*. Hubbard & Rimington (30) using tracer techniques found that both the nitrogen and the α -carbon atoms of labeled glycine were utilized specifically and with a high degree of efficiency in the biosynthesis of prodigiosin. The carboxyl-carbon of glycine was not utilized. Both carbon atoms of acetate were utilized specifically and to about the same extent.

The hypothesis of conversion of uroporphyrin to coproporphyrin to protoporphyrin by way of successive decarboxylations and dehydrogenations is receiving increasing support, although the evidence is still indirect. Two types of porphyrins exist in nature, the symmetrical type I being present in more than traces only in patients with congenital porphyria, and being represented by uroporphyrin and coproporphyrin; no protoporphyrin type I has ever been isolated. The type III porphyrins are represented by coproporphyrin and protoporphyrin, i.e., isomer #9. Coproporphyrin type III [Fig. 1 (III)] excretion is associated with acute porphyria, lead poisoning, sulfonal therapy, and alcoholic cirrhosis (31, 32). The biological occurrence of uroporphyrin of type III [Fig. 1 (II)] had been questioned (33), but indirect evidence of its presence is accumulating. Since this latter porphyrin

would be a reasonable precursor for protoporphyrin and would suggest what the possible structure of the natural monopyrrole might be (see above), the papers are cited on which the claims for its presence are based. Fourie & Rimington (34) in 1937 reported that cattle with ochronosis had a relatively high content of uroporphyrin type I and possibly some of type III, and Fischer & Hofmann (35) found a similar preponderance of type I uroporphyrin accompanied by a lesser amount of type III in the urine of patients with chronic porphyria. The pigment from the feathers of the Turaco bird was found by Rimington (36) to be copper uroporphyrin type III. In acute porphyria urine a colorless substance called porphobilinogen is excreted. Its properties suggest that it might be a precursor of uroporphyrin. Heating this urine in acetate buffer pH 5 for 30 min. at 100°C. brings about maximum conversion of porphobilinogen to uroporphyrin type III according to Grieg, Askevold & Sveinsson (37).

Gray & Holt (38) have identified the porphyrin excreted into the medium by *C. diphtheriae*, when grown on a medium low in iron, as coproporphyrin type III. Todd (39) was able to isolate considerable amounts of coproporphyrin type III from *Mycobacterium Karlinski*.

The identification of the isomeric types of porphyrins depends upon the melting point of their esters, a method that often leaves much to be desired. Solubility properties and adsorption spectra generally do not distinguish between them. Coproporphyrin type I and III may be distinguished on the basis of their fluorescence under certain conditions (40); uroporphyrins types I and III may be separated from each other on the basis that type III is extractable with ethyl acetate (41). The possibility that the infrared spectra of the isomers might differ significantly is suggested by the recent study of Gray & Neuberger (42) who report that the crystalline coproporphyrin esters of types I and III examined as a suspension or "mull" are readily distinguishable. It is to be hoped that this infrared method will find further application in isomeric analysis and will be modified for use with microgram quantities. X-ray diffraction studies of crystals should also be attempted to distinguish between these isomers.

The decarboxylation of uroporphyrin to coproporphyrin is compatible with chemical knowledge, since pyrrole acetic acids are readily decarboxylated. Support for such decarboxylation comes from the recent work on the isolation of porphyrins which contain carboxyl groups varying from 8 down to 2 per molecule. Grinstein, Schwartz & Watson were the first to isolate a heptamethyl ester porphyrin (43). Nicholas & Rimington (44) and McSwiney, Nicholas & Prunty (45) using paper chromatography found that there was a straight line inverse relationship between the rate of migration (R_F) of the porphyrin and the number of carboxyl groups on the porphyrin. On this basis they were able to demonstrate porphyrins containing 2, 3, 4, 5, 6, 7, and 8 carboxyl groups from the urine of porphyria patients.

Further suggestive evidence that the carboxylated porphyrins are precursors of protoporphyrin are presented in the studies on *C. diphtheriae*. It had been shown by Pappenheimer (46) that on low iron the production of porphyrin as well as of diphtheria toxin excreted into the medium was high; when the iron content of the medium was increased, both the excretion of porphyrin and the toxin were diminished. Studying the heme and porphyrin pigments, Rawlinson & Hale (47) observed that on a medium low in iron, the diphtheria bacillus excreted high concentrations of coproporphyrin type III at the same time that heme production was diminished. When the iron content of the medium was increased to a level sufficient to inhibit toxin and coproporphyrin production, the intracellular heme components, namely, cytochrome-*a* and *b*, but not *c*, rose five to ten-fold.

Studies of Grinstein *et al.* (48, 49) feeding N-labeled glycine support the idea that coproporphyrin I and III, uroporphyrin I, and stercobilin [Fig. 4 (XXVI)] are derived from a common pyrrole precursor which is most likely also the precursor of hemoglobin, i.e., protoporphyrin.

There are some data that might be considered not to favor the hypothesis that uroporphyrin is the precursor of copro- and protoporphyrin. Grinstein *et al.* (50) fed N^{15} glycine to a congenital porphyria and found that of the coproporphyrin type I excreted, a high proportion of it was labeled at first; the per cent of labeled coproporphyrin then rapidly decreased and was followed by an increase in the per cent of labeled uroporphyrin type I. These data suggested to these authors that uroporphyrin was derived from coproporphyrin. However, as pointed out by London (51) and by Gray & Neuberger (42), one is dealing here with the body as a unit and it would be necessary to know the rate at which these porphyrins are made, the rate of their excretion, and their content in the body stores before one could decide which compound preceded the other on the basis of the amounts labeled.

Rimington and co-workers (10), studying the incorporation of N^{15} into coproporphyrin type III in the diphtheria bacillus, found that the labeling in coproporphyrin was far lower than the labeling of intracellular heme and concluded that coproporphyrin type III might be formed by a synthesis independent of protoporphyrin synthesis. However, another explanation of this result might be that labeled glycine, present in limited amount, was used up very rapidly at first to form labeled heme and labeled coproporphyrin, and the coproporphyrin forming later from endogenous glycine would have diluted the labeled coproporphyrin already formed. The data presented were insufficient to decide this point [Hale *et al.* (108)].

Porphyrins in plants having the solubility and fluorescence properties of uro- or coproporphyrins have been found by Klüber (52) in legume root nodules where N_2 is fixed and where hemoglobin is also found. Goodwin, Koski & Owens (53) observed that the epidermal leaf cells in several species of vetch contained cytoplasmic bodies which were several times as large as the nuclei. These bodies fluoresced red. The absorption and solubility prop-

erties and the fluorescence spectra indicate that the fluorescing substance may be a porphyrin.

BILE PIGMENTS

These are "open chain" tetrapyrrole compounds [Fig. 4 (XXX)], lacking the α -methene bridge carbon atom which binds the tetrapyrrole into a ring porphyrin. The organic chemistry of the bile pigments has been reviewed by Fischer & Siedel (54). Since the bile pigments are open-chain tetrapyrroles

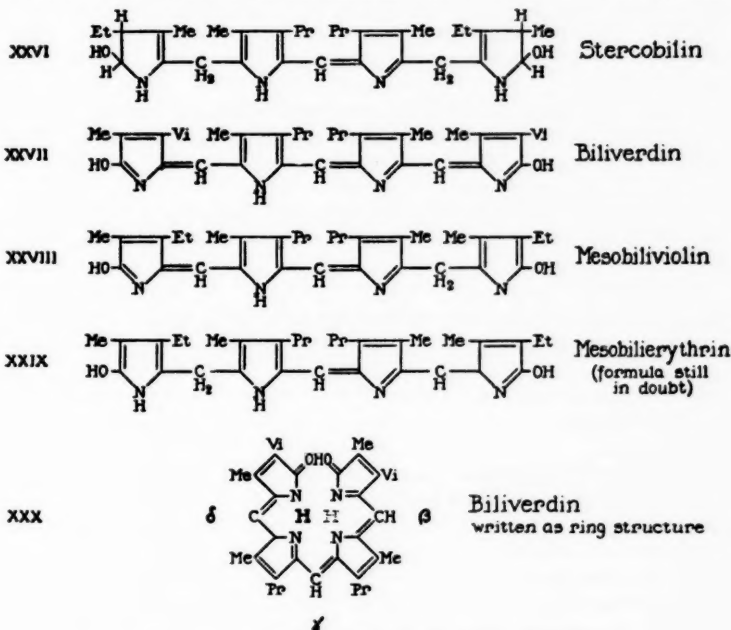


FIG. 4. Formulas of some bile pigments.

one might consider some of them to be precursors of the porphyrins. It is possible that porphobilinogen might be such a compound; this is a water-soluble diazo-reacting substance found in the urine of acute porphyrias which forms uroporphyrin type III on standing. The structure of porphobilinogen is, however, unknown.

In general, the bile pigments which have been isolated are considered to be derived from the decomposition of iron porphyrins (8, 26). London (51) has recently demonstrated that when labeled heme is injected into the blood stream of an animal, some 18 per cent can be isolated in the form of labeled

stercobilin from the feces during a nine-day period after injection. There is no biological evidence that free porphyrins, not in complex with iron, can give rise to bile pigments.

Using N^{15} labeled glycine, London *et al.* (56) discovered that stercobilin [Fig. 4 (XXVI)] was derived not only from the hemoglobin of aged red blood cells, but appeared in rather high amounts in the feces during the first two weeks after feeding the labeled glycine. Further experiments indicated that in normal humans, at least 11 per cent of the stercobilin was derived from a source other than that of the circulating blood and in a patient with congenital porphyria at least 31 per cent of the stercobilin was derived from noncirculating hemoglobin. Early labeling of coproporphyrin type I and uroporphyrin type I in addition to stercobilin was reported by Grinstein *et al.* (48, 49), indicating that these compounds were not derived by way of decomposition of red cell hemoglobin. London *et al.* (56) also reported that uroporphyrin type I and coproporphyrin type I reached approximately the same order of high labeling. Gray & Neuberger (42) examined the possibility that the early labeled stercobilin, especially high in porphyriurics, might be derived from the decomposition of type I porphyrins rather than from normal heme; such a stercobilin would be expected to be a different isomer, and have more carboxyl groups. However, the stercobilin isolated was identical with the normal one, elementary analysis indicating no more than two carboxyl groups, the visible and infrared absorption spectra and specific rotation values being the normal ones. In general, the data now available indicate that stercobilin is derived from the decomposition of heme, the heme source for the early formation of stercobilin being unknown at present (25).

In plants, bile pigments have been reported in two places. In root nodules of legumes containing hemoglobin, when plants are kept in the dark for an extended period, the hemoglobin breaks down, the decomposition of the heme being presumed to go via methemoglobin to biliverdin (8, 55).

In the chloroplasts of red algae and in the blue-green algae are present the crystallizable proteins, the phycoerythrins, and phycocyanins (8, 57). According to Lemberg, the prosthetic groups imparting the color to these proteins are bile pigments, phycocyanin containing mesobiliviolin [Fig. 4 (XXVIII)] as prosthetic group and phycoerythrin containing mesobilieriethrin (XXIX). Biliverdin (XXVII) is the bile pigment directly derived from heme by splitting out both the iron atom and the α -methene bridge carbon atom. Mesobiliviolin and mesobilieriethrin are related to biliverdin by addition of hydrogen atoms to the vinyl groups of biliverdin to form ethyl groups and by interruption of the resonating single-double bond system by further addition of hydrogen atoms.

These prosthetic groups of phycocyanin and phycoerythrin are firmly bound to their proteins, requiring rather drastic treatment with alkali or concentrated HCl to release them. Further studies on the character of the pigments and their binding linkages to the proteins would be desirable.

Haxo & Blinks (58) have recently studied the photosynthetic responses of chloroplast pigments other than chlorophyll-*a*. In the case of the red algae, phycoerythrin appeared to be the primary light absorber, the photosynthetic activity of the light absorbed by chlorophyll being minimal. The photosynthetic function of a pigment, low in the biosynthetic chain, is of interest from an evolutionary point of view and is discussed in a later section.

In considering the evolutionary development of the phycoerythrins and phycocyanins, it becomes important to know whether the colored prosthetic groups of these proteins originate from chlorophyll or from tetrapyrroles lower in the biosynthetic chain. From studies on the chemistry of phycoerythrin by Lemberg it became highly improbable that the pigment component could be a derivative of chlorophyll. Indeed, the side chain structure of the phycoerythrin pigment supported the idea, first proposed by Lemberg, that it was related to protoporphyrin. Further support for this viewpoint comes from the work of Siedel (59) who reported the isolation of glaukobilin by alkaline treatment of phycoerythrin. The side chains of the glaukobilin molecule differ from those of protoporphyrin and of the open-chain biliverdin [Fig. 4 (XXVII)], by having the two vinyl groups hydrogenated to ethyl groups. The identification of this glaukobilin as isomer 9 (i.e., of the same isomeric constitution as that derivable from naturally occurring heme) was made by x-ray crystallographic diffraction.

THE IRON BRANCH AND THE BIOSYNTHESIS OF HEME

The insertion of iron into protoporphyrin gives rise to the "iron branch" of the biosynthetic chain (Fig. 1). The iron metalloprophyrin or its modifications are the prosthetic groups of the cytochromes, peroxidase, catalase, hemoglobin, etc. A number of general reviews by Lemberg & Legge (8), Granick & Gilder (26), and Theorell (60) have discussed the properties and chemistry of these heme proteins. In connection with plants, the recent reviews of Scarisbrick (61) and of Goddard & Meeuse (62) are pertinent.

That iron is incorporated into the biosynthetic scheme at the level of protoporphyrin, and not into compounds below this level, is suggested by the following facts. No iron complexes of uroporphyrin or coproporphyrin are known to occur biologically. The open-chain tetrapyrrole pigments have not been found as iron complexes, with the exception of the choleglobin complexes [Lemberg & Legge (8)] which are obviously degradation products of heme. The studies of Granick & Gilder (26) on *Hemophilus influenzae*, an organism which needs iron porphyrins for growth, have shown that growth will occur if a porphyrin containing vinyl groups (i.e., protoporphyrin) is fed. This organism can only insert iron into a porphyrin bearing vinyl groups.

In addition to iron protoporphyrin, a few other modified iron porphyrins exist. It has been shown by Theorell (60) that the structure of cytochrome-*c* involves two thioether bridges between the apoprotein and the porphyrin, i.e., at positions occupied in protoporphyrin by the vinyl groups. Confirma-

tory evidence for the existence of the thioether link has now been obtained by Paul (63) who used Ag_2SO_4 in acetic acid to split this link. Studies by Warburg (64) and by Negelein (65) on the prosthetic group of cytochrome oxidase had revealed an iron porphyrin containing a formyl group as side chain. Warburg has suggested that this iron porphyrin might contain a phytol in its structure. Rawlinson & Hale (66), studying the heme of cytochrome-*a* from ox heart, have confirmed the presence of a formyl group by absorption spectrum studies.

Information on the cytochrome-*f* of Hill & Scarisbrick (67) is as yet available only in abstract form. This pigment is claimed to be characteristic of green plant tissue and was isolated from leaves with 50 per cent alcohol. The ratio of chlorophyll to cytochrome-*f* was given as 150:1. In liquid air, a sharp multiple banded spectrum of the alpha band of cytochrome-*f* was observed at 5,550, 5,510, 5,450 Å.

That some heme enzyme may be intimately connected with the photosynthetic mechanism is suggested by studies of Frenkel (68). At low partial pressures carbon monoxide was reported to inhibit photosynthesis of *Chlorella pyrenoidosa*. The inhibition was greatest at low light intensities and decreased with increasing light intensities, which suggests that a ferrous heme is involved. Since the release of oxygen by quinone and light was not inhibited by carbon monoxide, it is assumed that the oxygen-liberating catalyst was not affected, but that the acceptor for carbon dioxide reduction was inhibited.

Rosenberg & Ducet (69) have reported that spinach leaves contain an active cytochrome oxidase localized in the chloroplasts, in addition to a polyphenol oxidase.

THE MAGNESIUM BRANCH AND BIOSYNTHESIS OF CHLOROPHYLL

Protoporphyrin [Fig. 1 (IV)] normally occurs as the iron complex, iron protoporphyrin, or heme, in all plant and animal cells, as the prosthetic group of the heme enzymes. Even anaerobic cells may contain heme enzymes (26). Free protoporphyrin in general is detectable only in traces in cells. It cannot be detected in normal *Chlorella* cells. A *Chlorella* mutant, produced by x-rays, when grown in the dark on solid media containing agar, glucose, and inorganic salts, was found to develop a deep brown color [Granick (70)]. This brown color was due to protoporphyrin deposited in a granular form in large amounts in these cells. It was shown that this protoporphyrin was isomerically identical with the protoporphyrin of blood heme, i.e. isomer #9. No green or yellow pigments were produced by this mutant. Since heme is present in *Chlorella* cells in traces, the mechanism for protoporphyrin synthesis would necessarily be present, but only to an extent sufficient to provide the traces of protoporphyrin required for heme production. On the other hand, the absence of chlorophyll, which is normally made in large amounts in the cell, and the simultaneous appearance of large amounts of proto-

porphyrin, suggested that protoporphyrin was a normal metabolic precursor in the synthesis of chlorophyll.

Magnesium protoporphyrin.—From another *Chlorella* mutant which developed an orange brown color on solid media, magnesium protoporphyrin was isolated along with carotenoids, small amounts of protoporphyrin, and traces of a greenish pigment [Granick (71)]. The magnesium protoporphyrin was identified by its fluorescence, absorption spectrum, solubility properties, and qualitative test for the metal. Crystalline magnesium protoporphyrin was synthesized and its properties agreed with those of the compound isolated from this *Chlorella* mutant. The finding of magnesium protoporphyrin gives added support to the idea that protoporphyrin is a precursor of chlorophyll. At the same time it appears reasonable to consider that magnesium protoporphyrin is the next step after protoporphyrin in the synthesis of chlorophyll, thus giving rise to the magnesium branch of the tetrapyrroles. In analogy with the formation of iron protoporphyrin, it is probable that the insertion of magnesium is performed by a specific enzyme.

Magnesium vinyl pheoporphyrin a_5 (i.e., *protochlorophyll minus phytol*) [Fig. 1 (VI)].—From a yellow mutant grown in the dark, magnesium vinyl pheoporphyrin [Fig. 1 (VI)] was isolated [Granick (72)]. It was characterized by its absorption spectra and solubility properties and by its conversion into a number of derivatives. Characteristic absorption spectra of vinyl pheoporphyrin a_5 , vinyl chloroporphyrin e_6 trimethyl ester, pheoporphyrin a_5 , and pheoporphyrin a_5 oxime are described in this paper. Also described is a colorimetric method for Mg^{++} suitable for the range of 1 to 4 $\mu g.$ of magnesium.

Protochlorophyll is a pale greenish pigment containing two hydrogen atoms less than chlorophyll (see Fig. 1). It is found in traces in bean seedlings and in seedlings of a number of grasses grown in absolute darkness. It is also present in somewhat higher concentration in the cotyledons of pine seedlings and in the testa (the paper thin inner lining of the shell) of cucurbit seeds.

Noack & Kiessling (73) determined the porphyrin nature of this magnesium-containing pigment and its close relation to chlorophyll. Stoll & Wiedemann (74) expressed the idea which was later proved correct, that protochlorophyll is the magnesium complex of a vinyl pheoporphyrin a_5 compound esterified with phytol. Fischer, Mittenzwei & Oestreicher (75) started with chlorophyll, and by reducing it with iron in acetic acid according to the method of Noack & Kiessling, obtained on autoxidation vinyl pheoporphyrin a_5 , i.e., the porphyrin containing the cyclopentanone ring; the close similarity of protochlorophyll with pheophorbide a , the derivative of chlorophyll a , was thereby demonstrated. The structure of pheoporphyrin a_5 was proven finally by partial synthesis [Fischer & Oestreicher (76)]. That this pigment occurs as the phytol ester rests on indirect evidence which has only recently been obtained. Koski & Smith (77) found that the protochlorophyll isolated from etiolated barley by chromatography possessed a

magnesium content compatible with the assumption of a phytol ester, and Granick (72) found that the solubility property, i.e., extraction of protochlorophyll from ether with aqueous hydrochloric acid above 8N, was also compatible with the presence of phytol. The quantitative absorption spectrum of protochlorophyll has been determined by Koski & Smith (77).

The fact that protochlorophyll contains two hydrogen atoms less than chlorophyll has led to a number of speculations. It was suggested that protochlorophyll might be a precursor of chlorophyll or an oxidation product of chlorophyll, possibly a waste product, or that together with chlorophyll it might represent a reversible redox system functional in photosynthesis.

The evidence that protochlorophyll is the normal precursor of chlorophyll *a* is now at hand, and is based on two lines of evidence, namely: studies on action spectra; and the demonstration of a one to one correspondence between the disappearance of protochlorophyll and the appearance of chlorophyll. In 1945 Frank (78) found that the action spectrum in etiolated oat seedlings for the formation of chlorophyll resembled the absorption spectrum of protochlorophyll in solution, except that there was the expected slight shift of the spectrum to the red.

The action spectrum for chlorophyll formation has been studied more recently by Smith, Koski & French (79). They used albino corn containing only small amounts of carotenoids so that carotenoids would not interfere with the action spectrum studies. They found that blue light was the most effective part of the spectrum for the conversion of protochlorophyll to chlorophyll. This result was to be expected since the absorption of protochlorophyll in solution at 435 $m\mu$ is some ten times higher than at 623 $m\mu$. Direct evidence was obtained that the light absorbed by chlorophyll at 680 $m\mu$ was not involved in this conversion. A notion as to the extreme light-sensitivity of this reaction is given by the data of Koski & Smith (80). These workers found that when etiolated corn seedlings were illuminated at a temperature of 5°C. with 240 foot candles of light energy from a 40 watt white fluorescent lamp, then in 10 sec. some 56 per cent of the protochlorophyll was reduced to chlorophyll.

In connection with studies on the action spectra, Frank (78) concluded that in etiolated oat seedlings the carotenoids were not acting as a screen to filter out light in the blue region, otherwise the peak in the blue region of the action spectrum of protochlorophyll would not have been observed. These results were explained on the basis that protochlorophyll might be nearer the surface of the chloroplasts than were the yellow pigments. Results with normal etiolated corn and bean leaves by Smith, Koski & French (79) indicate that in these plants the carotenoids reduce the light action considerably by acting as a filter for blue light. It should be pointed out that the data of Frank also showed that some screening by the yellow pigments had occurred since blue light was only about equally effective with red light whereas without screening, the blue light could be theoretically ten times as effective.

The second line of evidence of conversion of protochlorophyll to chlorophyll was obtained by Smith (81) in a systematic study of photochemical and temperature effects on chlorophyll development. When etiolated barley seedlings were illuminated at 0° quantitative conversion of protochlorophyll to chlorophyll was found. At 0° no new protochlorophyll was produced either in light or dark. In dark-grown corn leaves, illuminated at 30, 120, and 240 foot candles with leaves at 5° and 18°C. it was shown that the rate of chlorophyll formation was roughly proportional to the light intensity indicating that the temperature at 5°C. was not a limiting factor. The reaction obeyed a second order curve (82).

Experiments of Smith & Koski (82) have shown that protochlorophyll is present not only in the etiolated plant but also in barley leaves during later stages of greening and in the mature leaves growing in the field. In etiolated seedlings exposed to either continuous or intermittent light, chlorophyll *a* is produced, and later chlorophyll *b* appears. Thereafter chlorophylls *a* and *b* increase in constant proportion to each other. A decrease in temperature decreases the rate of chlorophyll synthesis, but the ratio remains unchanged.

Chlorophyll formation in the absence of light.—A number of plants, especially among the unicellular green algae and seedlings of most conifer species, do not require light for the formation of chlorophyll. *Chlorella* and *Proteococcus*, when grown in the dark on nutrient media, have been shown by Meyers (83) to produce chlorophylls identical with those formed in the light, and equally effective in photosynthesis. Smith & Koski (82) studying chlorophyll formation in pine seedlings observed that germination of the seedlings at 17°C. resulted in dark green cotyledons with a normal ratio of chlorophyll *a*:*b*. Protochlorophyll was present in a ratio of 2 per cent to that of chlorophyll. Illumination of the dark-germinated seedlings had no effect on the quantities of chlorophylls *a* and *b* and protochlorophyll. Although in the dark the cotyledons become dark green, the developing leaves and stems of pine saplings, 20 inches tall, are nearly colorless, containing only traces of the chlorophylls and protochlorophylls. Bogorad (84) observed that embryos of *Pinus Jeffreyi* produced chlorophyll when germinated in contact with the megagametophyte, but when the embryos were excised and grown on tissue culture then the embryos produced little or no chlorophyll, the amount depending on previous length of contact with the megagametophytes during germination. When the green seedlings were grown in the dark, then exposed to light, and subsequently placed back in the dark, a significant loss of chlorophyll was observed, suggesting that light had induced the loss of the chlorophyll.

The biochemical conversion of protochlorophyll to chlorophyll.—This involves the addition of 2 hydrogen atoms to pyrrole ring IV. Since this reduction may occur in the dark in a number of plants, it is presumably brought about in these plants by an enzymatic reaction. In *Chlorella vulgaris* chlorophyll is produced in the dark. Certain mutants of *Chlorella* when

grown in the dark have been found by Granick (unpublished data) to lack chlorophyll, although possessing a deep yellow color due to the presence of carotenoids. When such yellow mutants are exposed to light they develop chlorophyll and can photosynthesize and therefore react like the higher plants in this respect. Here the process of enzymatic reduction of protochlorophyll is replaced by a photochemical process.

This photochemical process is of especial interest, since it brings about the addition of two hydrogen atoms to the photo-absorbing molecule, a change which involves a storage of energy. The questions arise as to whether all the energy involved in this reduction is supplied by the photons absorbed, or whether light is required merely as a trigger mechanism to permit some enzymatic reductive process to act, or whether a combination of these two mechanisms takes place. If the absorbed photons supplied all the energy, this would be akin to the process of photosynthesis. Smith (85) investigated the possibility that this photoreduction of protochlorophyll might be coupled with decomposition of water as in photosynthesis. No oxygen release during protochlorophyll reduction could be demonstrated even with a delicate phosphorescence quenching method for the detection of oxygen. Whether oxygen, coming from the water, is used up before it can be released as free gas, or whether only some intermediate steps toward the formation of oxygen are accomplished, would be difficult to determine.

The suggestion that protochlorophyll together with chlorophyll formed a redox system with protochlorophyll as the photoacceptor in normal photosynthesis was ruled out by the tritium experiments of Norris, Ruben & Allen (86) and more recently by the experiments of Smith & co-workers on the properties of protochlorophyll cited above. It is thus necessary to consider the photoreduction of protochlorophyll as a process independent of photosynthesis in which chlorophyll acts as photoacceptor.

That not merely the presence of chlorophyll, but also some factors, perhaps enzymatic links, must be developed before active photosynthesis will occur is suggested by the work of Davis (87) on certain green *Chlorella* mutants which fail to photosynthesize. A similar interpretation may be given to Smith's (88) finding that chlorophyll, derived from the protochlorophyll initially present in leaves, is not active in oxygen production immediately on its formation, but that a sojourn in the dark generates this capacity.

Chlorophyll c.—This pigment, discovered by Strain & Manning (89), along with chlorophyll *a*, represent the two tetrapyrrolic pigments of the chloroplasts of the diatoms, dinoflagellates and brown algae. Granick, in a preliminary note, (90) has recently identified chlorophyll *c* as a compound containing magnesium whose porphyrin possesses the cyclopentanone ring. This porphyrin is on an oxidation level with pheoporphyrin rather than with pheophorbide (i.e., pyrrole ring IV is not reduced) (see Fig. 1). It also contains an as yet unidentified chromophore group.

According to Strain (91), in diatoms and deep water brown algae the photosynthetic efficiency in the spectral range of 500 to 600 $m\mu$ approaches that in red light. Since Dutton & Manning (92) have shown that chlorophyll *c* absorbs much of the incident light in this spectral region, Strain believes that this pigment must be effective in the photosynthetic process.

Chlorophyll *a*.—Recent tracer studies on *Chlorella* by Salomon, Altman & Rosa (93) have shown that the α -carbon of acetic acid and the α -carbon of glycine are employed in the biosynthesis of chlorophyll [Fig. 1 (VIII)]. Since protoporphyrin is similarly labeled, this result supports the theory that protoporphyrin is a precursor of chlorophyll. *Chlorella vulgaris* was grown on a glucose, inorganic salt medium to which the labeled compounds in 0.01 *M* concentrations were added. The cells were grown in light and harvested after a week. The contribution of the α -carbon of acetate to the methyl pheophorbide isolated was 3.5 times greater than that of the α -carbon of glycine.

Arvanitaki & Chalazonitis (107) have studied the action of light on the pigmented neurons of *Aplysia*. The neurons of young *Aplysia* contain a series of chlorophyll-like and carotenoid bands. *In vivo* the chlorophyll-like pigment shows bands at 672, 611 and 560, the carotenoid bands being at 482 and 447 $m\mu$. It is not known whether these pigments are derived from the plankton, and what the functions of these pigments in the neurons might be.

Chlorophyll *b*.—This compound differs from chlorophyll *a* by substitution of $-\text{CHO}$ in place of a $-\text{CH}_3$ in pyrrole ring II. Whether the formation of this formyl group occurs at an early or at a late stage in biosynthesis is unknown. It is probable that chlorophyll *b* is functional in photosynthesis, although this has not been established.

That chlorophyll *b* is not essential for photosynthesis has been suggested by various experiments. Goodwin & Owens (94) had observed that in etiolated oat seedlings, when exposed to light, chlorophyll *a* was produced, and only after about 3 hr. was chlorophyll *b* detected. Schwartz (95) observed that in luteus seedlings of mutant corn, chlorophyll *a* preceded the synthesis of chlorophyll *b* by four days when grown at 85°F. The formation of chlorophyll *b* was strongly influenced by temperature. At 62°F. the retardation in chlorophyll *b* formation extended over a period of 11 to 14 days. Highkin (96) studying a barley mutant, chlorina #2, which could photosynthesize, reported that it seemed entirely lacking in chlorophyll *b* content, although its chlorophyll *a* content was normal.

Magnesium metabolism.—Smith (97) has summarized his extensive studies on changes in the ether-soluble magnesium compounds of barley seedlings as effected by light and temperature. The photochemical and thermochemical reactions leading to the synthesis of chlorophyll appear to operate in some kind of integrated fashion during greening. They cannot as yet be separated from phenomena involved in growth and development of the chloroplasts themselves. Illumination of etiolated barley seedlings resulted in the incorpo-

ration of inorganic magnesium into newly-forming chlorophyll. During the first 2 hr. of illumination at 19°C. the increase in total ether-soluble magnesium was greater than the increase in chlorophyll-magnesium, but thereafter the increases in these two fractions nearly coincided and reached a limit of about 190 $\mu\text{g.}$ per gm. dry weight at the end of 45 hr. of illumination. Illumination of etiolated barley at 0°C. led to very slight increases in total fat-soluble magnesium reaching a maximum at the end of 2 hr. After 43 hr. of illumination at 7°C. the total ether-soluble magnesium was 80 $\mu\text{g.}$ and chlorophyll-magnesium was 57 $\mu\text{g.}$ per gm. dry weight.

Gilbert (98) noted that the peroxidase of magnesium-deficient tung leaves was increased over that of normal tung. He suggested that the high peroxidase might result from an accumulation of chlorophyll precursors, presumably protoporphyrin, which would be converted to heme, the prosthetic group of peroxidase.

Data on other mutants.—V. Euler (99) studied barley mutants, induced by x-rays, that were deficient in chlorophyll. On examination ten days after germination it was noted that, for equal wet weights, the normal seedlings had about six times more catalase activity than the mutants deficient in chlorophyll. This lowered activity was shown not to be due to the presence of a catalase inhibitor specifically generated in the tissues of the mutants.

Schwartz (100), using mutants of maize, found a close correlation between plastid size and pigment development in cream-white types (i.e., lacking chlorophyll but containing some yellow pigments), in virescent types (containing some chlorophyll *a* and *b*), and in pale green mutants, but not in the luteus types (containing some green and some yellow pigments).

Highkin (96) determined the chlorophylls in a number of chlorina stocks of barley. It was found that chlorina #3 in linkage group 7 had a significantly higher *a* and *b* content than the normals, and that the ratio of *a:b* was normal. Chlorinas #4 and #5 were not significantly different from normal. Three other chlorinas were low in chlorophyll, although still possessing a normal ratio of *a* to *b*. One extreme chlorotic type and one albino were completely lacking in chlorophyll. Chlorina #2, although possessing a normal chlorophyll *a* content, was completely devoid of chlorophyll *b*.

Smith & Koski (82) found that one of the albino strains of corn did not turn green to an appreciable extent because an excessive rate of destruction of chlorophyll was occurring in the light. Several of the virescent corn mutants were found to be partially defective in the rate at which they made protochlorophyll, but they possessed the mechanism for the continued production and conservation of chlorophyll.

Effect of streptomycin on chlorophyll production.—In 1948 v. Euler, Bracco & Hiller (101) reported that seeds, germinated on filter paper wet with a streptomycin solution greater than 2 mg. per cc., led to the development of completely colorless coleoptiles and first leaves. The seedlings investigated were barley, lettuce, rye, spinach, and radish. With less concentrated solu-

tions, only the tips of the leaves became green. Streptomycin appeared to retard or arrest chlorophyll formation in developing leaves, but chlorophyll, already formed in leaves before treatment, was unaffected. The authors suggested the hypothesis that streptomycin reacts with nucleic acids and nucleoproteins of the chondriosomes, modifying their differentiation and division. De Ropp (102) observed that streptomycin changed crown gall tumors from green to white, and inhibited the growth of sunflower root and tumor tissue.

Provasoli, Hutner & Schatz (103) studying the action of streptomycin on *Euglena gracilis* var. *bacillaris* found that streptomycin brings about an irreversible loss of chloroplasts. The colorless organisms remain colorless and devoid of chloroplasts throughout succeeding generations. However, the paramylum granules continue to be formed, and the orange eye spot, although smaller than normal, also remains. The significance of these findings in terms of cytoplasmic inheritance has been discussed by Granick (104). More recent unpublished experiments by Hutner have shown that var. *bacillaris* and *urophora* of *E. gracilis*, when exposed to a temperature of 32 to 35°, lose their chloroplasts permanently.

Bogorad (105) germinated seeds of *Pinus Jeffreyi* on agar containing streptomycin. At 0.2 per cent of streptomycin the cotyledons of almost all seedlings developed without chlorophyll, although the cotyledons of these seedlings normally form chlorophyll in the dark. When the cotyledons were removed from their megagametophytes and placed on a medium devoid of streptomycin, then they would turn green, but only in the presence of light. This would suggest that the enzyme system which served to reduce proto-chlorophyll in these cotyledons in the dark was damaged by streptomycin. Umbreit (111) has found that streptomycin inhibited concurrent oxidation of pyruvate and oxaloacetate in *E. coli* and animal tissue preparations. Resistant variants of *E. coli* survived apparently because they could by-pass the pyruvate-oxaloacetate condensation.

Criteria of identification of porphyrin compounds.—The problem of identification of small amounts of pigments from mutant *Chlorella* has led to a scrutiny of the methods and criteria on which the establishment of identity has been based. The high molecular weight, complex structure, and lability of the tetrapyrroles make their identification difficult. Since these pigments vary in molecular weight from 600 to 900 the customary identification procedures of organic chemistry are not completely satisfactory. For example, carbon analysis may deviate by plus or minus 2 carbon atoms. In one case, Fischer's surmise as to the structure of deuteroporphyrin was wrong and remained uncorrected for several years because it was based on structural interpretation from carbon analysis alone.

Melting point determinations of these pigments must also to be scrutinized carefully, even though the melting points of the mesoporphyrin esters form the present basis for distinguishing between the type I and the type III

isomeric porphyrins. For example, the melting points of mesoporphyrin type III ester reported over a period of years was 206 to 216°. Indeed, on the basis of melting points, Fischer at one time claimed the presence of two isomeric hemes in hemoglobin, but retracted this notion a year later. Melting point determinations of the porphyrins generally have been carried out in melting point glass capillaries. The complexity of melting of porphyrins when carried out under a hot stage microscope using crossed Nicols has been described by Granick (70). The crystals were crushed between two cover slips and streaks of the crushed crystalline fragments were observed under the microscope. As the temperature was raised and approached incipient melting, in the region of the streaks tiny crystals developed and grew with their long axis oriented parallel to the direction of the streaks. After melting had begun, within the droplets could be seen numerous small crystals rapidly forming and melting. Only when the temperature had risen 2 to 4° above the incipient melting point had all birefringent material disappeared. A comparison of melting point behavior under the above conditions should make more certain identifications based on melting points. In general it is apparent that no one criterion of identity offers complete proof, and it is only possible to approach greater certainty if several independent methods are used.

Methods of identification based on the crystallinity of the porphyrins are precluded in the case of some of the tetrapyrrole pigments isolated from mutant *Chlorella*, because of the minute concentrations present, and because of their lability. A technique of identification based on the properties of partition between two immiscible solvents i.e., between ether and aqueous hydrochloric acid or ether and aqueous alkaline buffers, and also based on absorption spectra, has been utilized by Granick (71, 72). The characteristic spectra of the porphyrins, their intense absorption, and the relative ease with which measurements may be made in a Beckman spectrophotometer make this the method of choice for identification. For example, the complete spectrum of protoporphyrin may be obtained with 0.3 mg. of material and with a semi-micro cell even 0.03 mg. is sufficient for identification. Comparison with a known compound is then made of wave length positions of the sharp band maxima and also of the relative heights of the bands to each other.

In general, this spectroscopic method will reveal the presence of resonating side chains i.e., side chains attached to the ring by alternating single-double bonds such as a vinyl, aldehyde, ketone, or acid group. Of great value are the published spectroscopic data on many of the compounds that Fischer and co-workers synthesized, so that certain correlations of chemical structure with characteristic spectral properties can be made (26). However, the absorption spectra do not indicate side chains which lack resonance. For this reason, the solubility properties or rather partition coefficients have been utilized. Chromatographic methods may also be used.

By studying these two properties, absorption spectrum and solubility,

a porphyrin may be identified in many instances with a reasonable certainty. (The kind of isomer can as yet only be identified from preparations of crystalline derivatives.) By preparing one or more derivatives which will reveal themselves by a characteristic change in absorption spectrum, additional proof of identity may be obtained. At the same time, the changed solubility properties of these derivatives can also be determined. For example, in the identification of vinyl pheoporphyrin a_8 (72), the presence of the C=O group in the cyclopentanone ring was confirmed by preparation of the oxime, which had a different and characteristic absorption spectrum. A characteristic reaction is the splitting open of the ring in methyl alcoholic HCl to form a trimethyl ester with a marked change in absorption spectrum which confirmed the presence of the cyclopentanone ring. By reduction of the compound with colloidal palladium plus hydrogen a shift of all the band maxima by 4μ to the blue was observed, indicating the presence of only one and not two vinyl groups.

EVOLUTION AND THE BIOSYNTHETIC CHAIN OF CHLOROPHYLL

The biosynthetic chain of chlorophyll has been suggested [Granick (106)] to represent a kind of evolutionary pathway. As outlined in Figure 2, the biosynthetic chain is composed of a series of reactions that involve specific enzymes and specific substrates and are presumably gene-controlled. Such a series of reactions might be conceived of as originating in a stepwise manner, the lowermost steps being formed first and the higher steps evolving later. The survival of a biosynthetic chain would depend on the ability of the end product of a chain to function effectively in the economy of the cell. The evolutionary development of a biosynthetic chain would depend on the ability of each newly created end product to function effectively. For example, according to this hypothesis the biosynthetic chain of chlorophyll (Fig. 1) would have its origin in the small molecules of the metabolic milieu such as glycine and acetic acid. From these small building blocks would be formed, in a stepwise manner, the more complex colored molecules such as the dipyrroles, tetrapyrroles, porphyrins, etc. Each of these colored molecules would at some time be the end of the chain and would have a specific function. At some time, let us say, protoporphyrin was the end of the chain and a change occurred to convert this compound to magnesium protoporphyrin; then the function of protoporphyrin might be lost, but the mechanism for the formation of protoporphyrin would still be retained because it was now an intermediate in the formation of magnesium protoporphyrin, i.e., an intermediate or precursor for a compound higher up the chain.

That certain of the compounds along the biosynthetic chain might still retain their original (vestigial?) functions is suggested by the following data: When protochlorophyll is illuminated with visible light it is reduced to chlorophyll a ; this reaction might be considered as a kind of protosynthesis in which the energy of sunlight is used for reduction. Chlorophyll

c, present in brown algae, is a pheoporphyrin-like compound (i.e., it is more closely related to protochlorophyll than to chlorophyll), and this pigment is believed to be functional in photosynthesis. The phycoerythrins of red algae, whose prosthetic groups are open chain tetrapyrroles, have been demonstrated to be highly active in photosynthesis. The three compounds mentioned are related to the biosynthetic chain of chlorophyll, and all seem to possess a photosynthetic function. It might be considered that at a rather early stage in evolution, when the pyrrole compounds had attained a sufficient complexity to absorb visible light, they already possessed a photosynthetic function. The further development of the biosynthetic chain would then represent modifications of the biochemical system to carry out the photosynthetic function with greater efficiency. Other viewpoints on the evolution of photosynthesis have been recently reviewed by Van Niel (109). Smith (110) has written a comprehensive review on the photosynthetic activity of pigments other than chlorophyll *a*.

SUMMARY

Advances of the last few years make possible the formulation of a scheme for the biosynthesis of chlorophyll. The biosynthetic chain of chlorophyll is presented in Fig. 1, and is intended to summarize the principal data and hypotheses covered in this review.

As learned from tracer studies, the biosynthetic chain begins with small building blocks, namely acetate and glycine molecules, that are part of the basic metabolic milieu. The acetates, via the citric acid cycle, are postulated to give rise to 4 carbon units, two of these units combining with glycine to form a pyrrole of the hypothesized structure, formula I. It is further envisaged that these pyrroles unite with others like it to form uroporphyrin in a series of *n* steps.

Formula II summarizes diagrammatically the probable origin of the atoms in uroporphyrin. Eight glycine molecules and eight 4-carbon units (possibly succinic semi-aldehydes) would be required. The nitrogen and carbon atoms, ringed together, are both derived from the same glycine molecule. Each ringed carbon atom, which serves to bind two pyrrole rings together, is derived from the α -carbon atom of another glycine molecule via a one carbon unit, in the reactions of which folic acid and vitamin B₁₂ may be involved.

Decarboxylation of uroporphyrin leads to the formation of coproporphyrin (III). Subsequent oxidation then converts coproporphyrin to the molecule protoporphyrin. In a *Chlorella* mutant devoid of chlorophyll and carotenoids, large amounts of protoporphyrin are formed.

From protoporphyrin (IV) two main branches arise. When iron is inserted into the center of the protoporphyrin ring, iron protoporphyrin (heme) is formed, this metalloprophyrin being the prosthetic group of various heme enzymes. This may be considered as the iron branch of the scheme. When magnesium is inserted into protoporphyrin, then magnesium protoporphyrin

is formed; this compound has been found in another *Chlorella* mutant. Then a number of stepwise changes must be postulated to occur to this molecule, one change being the reduction of one of the vinyl side chains to an ethyl side chain; other steps involve the oxidation of one of the propionic acids, the esterification of this acid with methyl alcohol and cyclization to form the cyclopentanone ring. These changes then lead to the formation of magnesium vinyl pheoporphyrin a_3 (VI) a compound found to be present in another *Chlorella* mutant. In the next step, the second propionic acid group is esterified with the C_{20} alcohol phytol, thus forming protochlorophyll. The reduction of protochlorophyll at pyrrole ring IV yields chlorophyll a (VIII). Possible relations of several other tetrapyrroles, discussed above, are also indicated in this scheme.

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RESPIRATION

THE PASTEUR EFFECT IN PLANTS

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Since the reviews of the Pasteur effect by Dixon (1) and Burk (2), general interest in this problem seems to have declined, possibly because Burk assembled such a bewildering array of possible explanations of the effect as he defined it. Thomas (3) is one of the few plant physiologists to discuss it fully in a textbook which is rather unusual in serving as a platform for interim publications of the work of his school. The present review will deal only with the Pasteur effect in plants and in particular will attempt to clarify a confused position and to discuss the concept of oxidative anabolism in the light of some recent papers.

DEFINITIONS AND SYMBOLS

Respiration (R).—For the purpose of this review we shall mean by Respiration (R) the complete oxidation of carbohydrates to carbon dioxide and water, molecular oxygen serving as the ultimate electron acceptor.

OR.—OR will mean [following Blackman (4)] the carbon dioxide production in respiration, used as a measure of carbon loss by this process. As there has been little support for the views of Boysen-Jensen and Lundsgaardh [Turner (5)], we shall assume that a carbohydrate-degradation process, glycolysis, is a part of respiration proper (6, 7).

Glycolysis (GL).—There has always been confusion over this term, which originally meant the production of lactic acid from glycogen and is sometimes used as a synonym for fermentation (7). In Blackman's formal catalytic scheme for apple respiration (4), glycolysis signified the conversion of reactant C (some form of active sugar) to reactant D, the substrate for the last stages of sugar breakdown which has alternative fates in air and nitrogen. However, biochemical evidence suggests that the formation of C (by phosphorylation) is linked with the later stages of sugar breakdown through a phosphate cycle. Moreover, while Blackman pictured D as including compounds of formula $C_3H_6O_3$, $C_3H_4O_3$, and C_2H_4O , we prefer to regard glycolysis strictly as including an oxidation occurring within the sugar molecule or its products. We therefore define glycolysis (in plants) as the degradation of hexose by a series of reactions involving an oxido-reduction, with the production of a simpler substance (D), which has alternative fates in air and nitrogen. It does not seem premature to picture this substance as pyruvic acid or acetaldehyde and we have abandoned the term triosis (5) as no longer a suitable synonym for glycolysis as now defined. Equations for glycolysis would therefore be: $-2Co + C_6H_{12}O_6 \rightarrow 2C_3H_4O_3 \rightarrow 2C_2H_4O + 2CO_2 + 2CoH_2$ where Co is a hydrogen acceptor, possibly coenzyme I. The reduced acceptor is

regarded as undergoing oxidation by different means in air and nitrogen. We must also assume that glycolysis in higher plants, as in yeast, involves phosphorylation and dephosphorylation.

Fermentation (F).—Fermentation (F) means the degradation of carbohydrate into two or more simpler molecules by processes not requiring molecular oxygen; it differs from though it includes glycolysis in that its end products either accumulate in or escape from the cell. The most frequent type is alcoholic fermentation, but some plants produce carbon dioxide and lactic acid during fermentation and there may be other products so far unidentified. We agree with Goddard (8) in preferring the term fermentation to the alternatives anaerobic respiration and intramolecular respiration, and since the term fermentation is no longer applied only to microorganisms we see no need to replace it with Thomas's rather narrower *zymasis* (3), which he defines as the process which causes alcohol and other products of *zymase* cleavage to accumulate in plant cells. We shall use the following symbols:

F_n —fermentation in the complete absence of oxygen;

NR—the carbon dioxide production in the absence of oxygen;

ALC—the alcohol produced by F, and

F_{o_2} —fermentation going on in some concentration of oxygen, not necessarily air, but referred to for convenience as aerobic fermentation.

It has long been known that the quotient NR/ALC generally exceeds unity in plants and it is probable that some of the carbon dioxide produced by an apparent fermentation really arises by the decarboxylation of organic acids, or from proteins. And in a great many of the papers on the Pasteur effect it is tacitly assumed that the fermentation is of the alcoholic type. Thomas (3) has pointed out the dangers of this assumption and some examples will serve to show how much remains to be done on the biochemistry of the fermentations in higher plants. Apart from certain difficulties in the determination of the products, there is always the need to guard against bacterial infection in experiments of this kind.

Derby & Goddard (9) have recently shown that the fungus *Myrothecium verrucaria* has a high rate of respiration but produces no carbon dioxide in either pure nitrogen or hydrogen; nor apparently does it produce organic acid under these conditions, for no carbon dioxide output was obtained in a medium containing potassium bicarbonate. Phillips (10) has shown that young barley seedlings carry out a basic alcoholic fermentation plus an extra carbon dioxide production which occurs mainly in the first few hours of anaerobiosis. Only a part of this extra carbon dioxide is accounted for as arising from bound carbon dioxide displaced by lactic acid. Older barley plants produce little alcohol and much extra carbon dioxide in fermentation. Fermentation in young rice seedlings is of the pure alcoholic type, but the fermentation resembles that in barley by the time the first leaf has emerged. In a later paper (11) she brings out the important point that for barley roots the carbon dioxide in excess of the alcohol formed probably arises not from carbohydrates but from either proteins or organic acids. Thomas & Fidler

(12) also showed that for two varieties of apple, supplementing a true alcoholic F there was excess carbon dioxide (NR_{RES}) amounting to from 25 per cent to 50 per cent of NR. Fidler (13) has now shown this NR_{RES} to arise from malic acid possibly by its complete oxidation rather than by decarboxylation. Such oxidation in anaerobiosis would involve the production of reduced compounds for which no evidence has yet been obtained. Nance (11) showed that NR was much greater when carbon dioxide-free air was led over a tissue than when it was enclosed in a Warburg respirometer. She supports the suggestion of Steward & Street (14) that there is more rapid decarboxylation when the gas phase lacks carbon dioxide. This hypothesis requires further testing with bulky tissues as with tissue slices, as it means that the choice of apparatus may markedly affect quantitative determinations of NR, or may change its origin.

Lactic fermentations may prove to be of more frequent occurrence in plant tissues than is generally realised. The acid is presumably produced by the reduction of pyruvic acid, this being an alternative fate to its decarboxylation. It is therefore important to measure not only the ratio of carbon dioxide to alcohol, but also to prepare, as far as possible, a carbon balance sheet for fermentation. Apart from loss of sugar due to lactic fermentation, with no corresponding metabolic carbon dioxide production, there is also the possibility, noted by Boswell & Whiting (15), of the increase in acidity (due to lactic acid) causing the release of bound carbon dioxide, the amounts of which may be large in bulky tissues.

Schneider (16) has shown that small quantities of lactic acid are produced aerobically in some leaves, tubers, roots, rhizomes, and seedlings. He could, however, establish no clear change in lactic acid production when the tissue was transferred from air to nitrogen, using carrot, potato, and bean leaves. The sharp maximum in the production of lactic acid in seedlings which occurs just before the breaking of the testa was not due to the lack of oxygen in the young tissues. He did not test the possibility that it was due to high internal carbon dioxide.

In spite of Schneider's results with potato, we now have clear evidence that this tissue, in some varieties at least, carries out a lactic fermentation. It has long been known to produce little alcohol, although in fermentation there is a marked production of carbon dioxide. Wetzel's (17) suggestion that this was accompanied by lactic acid has been confirmed by Saifi & Barker (18) who showed that potato tubers of low sugar content produce carbon dioxide, lactic acid and an alcohol-soluble intermediate compound, while alcohol does not begin to accumulate until the tissues have been for twelve days in nitrogen. During the fermentation, sucrose concentration falls, but not sufficiently to account for the total fermentation. Anaerobic metabolism in the potato, and possibly in other higher plants, may have some resemblances to that in certain fungi. In this group, lactic acid production is with a few exceptions confined to the *Mucoraceae*, chiefly to *Rhizopus*. These species produce some alcohol and many organic acids in fermentation, but there is a

40 per cent sugar conversion to lactic acid. In the strain *Rhizopus MX* there is evidence for the existence of a double fermentation [Foster (19)], expressed by the equation: $-C_6H_{12}O_6 \rightarrow C_2H_6O_3 + C_2H_5OH + CO_2$. Foster's account of the lactic fermentations by fungi will clearly be of value for comparative purposes.

It is, however, certain that in many higher plants fermentation is predominantly of the alcoholic type, although the quotient NR/ALC is rarely the theoretical 0.95. As Thomas (3) has shown, there is nearly always a small production of acetaldehyde accompanying the alcohol, and the ratio aldehyde to alcohol does not remain constant during the progress of fermentation. Thomas (20) and Thomas & Fidler (21) have succeeded in bringing about "CO₂-zymasis" and "HCN-zymasis" in aerobic apples by increasing the concentrations of these two substances and under these conditions the ratio aldehyde/alcohol is not that of normal fermentation.

The rate of fermentation, measured by carbon dioxide production rarely remains constant with time. Sometimes it falls sharply to zero, as in the avocado (22) and excised barley embryos (23). Sometimes, as in lemons (24), it drifts down very slowly, but remains well above the OR for as long as 23 days. In the apple (4), such a drift is more pronounced and its form varies with the "physiological class." In some seedlings (25) there is a rise followed by a fall in NR rate, while for carrot, rising rates have been recorded for whole roots (26) as well as for tissue slices (27). It was the marked change in NR rate that led Blackman to disregard earlier records of the NR/OR ratio and to determine NR by extrapolating to zero time in nitrogen. There are wide differences in the degree of susceptibility of plant tissue to a period of anaerobiosis and there is some evidence that during the drift in NR there may be changes in the nature of the fermentation. Thus, Wetzel (17) reported that for carrot tissue the ratio carbon dioxide to alcohol was at first 1.0, but soon fell away and reached .51 after 64 hours. On return of a tissue from anaerobiosis to air there is often a temporary stimulation of OR, but for some tissues at least (4, 27) long exposures to nitrogen have no permanent disturbing influence upon the metabolism.

Extinction point (E.P.).—This has been defined by Blackman as the lowest concentration of oxygen which just extinguishes all aerobic fermentation. It should therefore be determined by measurement of alcohol and other fermentation products, but this has only been done by Thomas & Fidler (28) and by Ranson (29), with results shown in Table I. For apple fruits and *Rhododendron* leaves the carbon dioxide production is at a minimum at the E.P. (sometimes called the critical oxygen concentration). This is not always so and the assumption (24, 26) that carbon dioxide production may be used to determine E.P. is not justified. In the seedlings of vegetable marrow, buckwheat, and wheat (29, 30), for instance, the minimal total carbon dioxide production was at zero oxygen concentration.

Extinction points for a few tissues have been determined by finding the lowest concentrations of oxygen in which the respiratory quotient remains at

TABLE I

CARBON LOSS BY MEASUREMENT OF OR, NR AND ALC COMPARED
IN NITROGEN, AIR, AND AT THE EXTINCTION POINT

Species	C loss in N ₂	C loss in N ₂	Extinction Point Per- centage O ₂	Ref.
	C loss in air	C loss at EP		
(a) Pasteur effect present				
<i>Rhododendron</i> leaves	4.0	5.3	1.8	(29)
<i>Pisum sativum</i>				(29)
edible pea, germinating seedling	4.8-6.1	—	—	
<i>Pyrus malus</i> , fruits				
Bramley seedling (a)	2.1	2.7	1.3	(3, 28)
Bramley seedling (b)	3.6-4.1*	—	—	(12)
Newtown pippin	2.9-3.2*	—	—	(12)
<i>Rumex</i> sp., leaves	1.3-1.4	—	—	(29)
<i>Cucurbita lepo</i>				(29)
germinating seeds, marrow	1.0	1.5	2.0	
(b) Pasteur effect apparently absent				
<i>Fagopyrum esculentum</i>				
Buckwheat, germinating seeds	0.5	1.0	1.5	(29)

* Allowing for NR_{RES}.

1.0; they range from 1 to 5 per cent oxygen (26, 30) except for seedlings of wheat and rice, for which Taylor (31) records exceptionally high figures, between 10.4 and 20 per cent. As in these experiments the respiratory quotient fell again to 0.85 in 50 per cent oxygen (being 1.0 in air) the figures for E.P. require confirmation, as do all in which extinction of alcohol production has not been demonstrated.

Thomas & Fidler (28) have shown that for apples the E.P. shifts to higher concentration of oxygen as the apple ages and old apples may produce alcohol even in 100 per cent oxygen. High figures for the E.P. may be obtained for bulky tissues and for germinating seeds still enclosed in the testa, where resistances to diffusion may limit the access of oxygen to the internal tissues. The E.P. shift in ageing apples may therefore be due to increased resistance in the skin tissues rather than to a breakdown of the oxidation system [Hackney (32)].

Pasteur effect (P.E.).—Most writers today use Dixon's definition (1) which runs . . . "The Pasteur effect is the action of oxygen in diminishing carbohydrate destruction and in suppressing or decreasing the accumulation of the products of anaerobic metabolism." Burk (2) has criticised this definition in his colloquial review and on three grounds—all inadequate:—(a) That there are few experimental determinations of carbohydrate loss, (b) that in aerobic cells anabolic processes with carbohydrates as substrate may

often go on fast enough to mask the conserving effect of oxygen, and (c) that the diminution of carbohydrate destruction does not always accompany suppression of fermentation. Burk's use of the more general definition of the Pasteur effect—that it is the suppression of fermentation by oxygen—together with Dixon's retention of the second half of his definition, has led to a good deal of confusion in the literature. Dixon himself says

it is evident that the first characteristic of the definition of necessity involves the second However, decrease in the formation of anaerobic cleavage products does not necessitate decreased carbohydrate catabolism. It is thus merely redundant to add the second quality to the definition, although it is this quality which is often mainly accentuated.

As examples of the confusion mentioned, we may refer to Platenius (30) who quotes Dixon's definition, but uses Burk's for he states . . . "the Pasteur effect comes into play whenever the storage atmosphere contains small quantities of oxygen." Merry & Goddard (33) obviously use Dixon's definition, but nevertheless state "the inhibition of F by oxygen is known as the P.E.," and they go on to distinguish between inhibition of F and oxidation of its products. Throughout this review we shall adopt the hypothesis that suppression of fermentation is due to the diversion of the products of glycolysis from fermentation to respiration (Burk's "pyruvate or acetaldehyde shunt"). What we are interested in here is not so much the existence of this shunt as its possible effect on the rate of carbon loss. We therefore adopt Dixon's definition in principle, but define the P.E. more strictly as follows:—The P.E. is operating in a respiring plant cell, if, on passage of the cell from that oxygen concentration known as the E.P., or from air or some higher concentration of oxygen, to anaerobiosis, the carbon loss is rapidly increased (Fig. 1).

This definition is rather more than a sharpening of Dixon's, as it allows us to bring out the important fact that both decrease and increase in oxygen concentration from the E.P. leads in some plants to an increased carbon loss. When this dual effect is present, there is a P.E. and yet there may be no change in carbon loss on transfer from air to nitrogen (Fig. 1). Thus a P.E. is really a conserving effect of oxygen on carbon under strictly defined conditions. We have adopted an experimental approach in the definition because on the reverse passage (nitrogen to air) the P.E. may be masked by the after effect [so called repayment of oxygen debt (11)].

Although it is not mentioned in the definition, it may be assumed that wherever the P.E. operates, oxygen decreases or suppresses the accumulation of the products of F. Our use of the word carbon instead of carbohydrate meets Burk's second criticism.

The Meyerhof quotient and the NR/OR quotient.—The Meyerhof quotient is defined by Warburg (for yeast) as $Q_{N_2}^{CO_2} - Q_{O_2}^{CO_2} / -Q_{O_2}$, that is, in our terminology, $NR_n - NR_{O_2} / OR_{O_2}$, or the decrease in the NR of fermentation divided by the oxygen uptake, which is assumed to be equal to the carbon

dioxide output in respiration with carbohydrate substrate. Because, per mole glucose, F produces 2M and R, 6M carbon dioxide, there is no P.E. if the Meyerhof quotient equals one third; when it is greater than one third, the P.E. is operating. This quotient is of little value in most work on plant

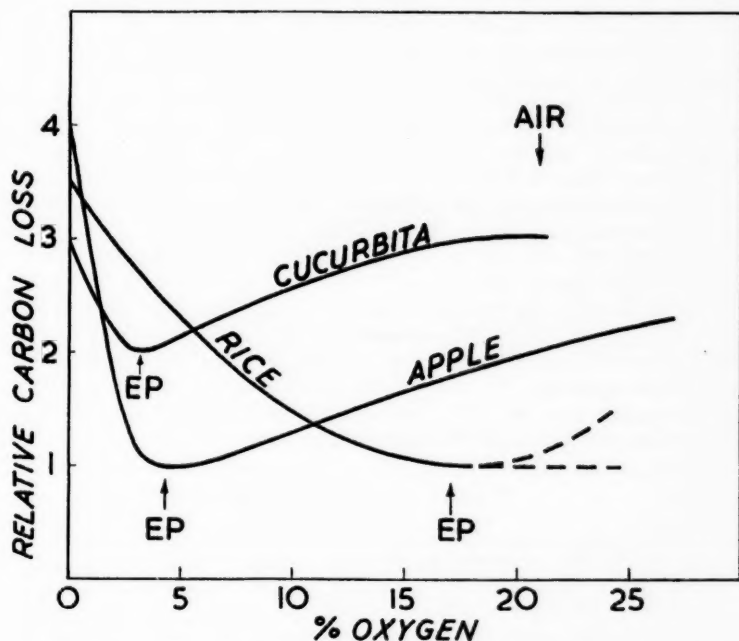


FIG. 1. Diagrammatic representation of some effects of oxygen concentration (on both sides of the E.P.) on carbon loss, illustrating three types of P.E.

tissues with their low extinction points and for them it is usually replaced by the quotient NR/OR , where these symbols represent rates of carbon dioxide production. The same quantitative considerations hold for this quotient as for the Meyerhof quotient, so long as the fermentation is entirely or predominantly of the alcoholic type.

EVIDENCE FOR THE EXISTENCE OF THE PASTEUR EFFECT IN PLANTS

Demonstration of greater carbon loss in nitrogen by carbohydrate analysis.—The direct method for demonstrating a P.E. is carbohydrate analysis in nitrogen and air, coupled with evidence showing to what extent carbohydrate loss is due to its destruction rather than to syntheses of other substances.

To the reviewer's knowledge, this method has so far been used only for yeast and apples. Gottschalk's (34) carbon balance sheet for yeast supplied with glucose (Fig. 2) provides clear evidence of the existence of the P.E. . . . "In the presence of oxygen (or air) the total amount of glucose used up is diminished by 30 to 45 per cent as compared with the consumption of glucose under anaerobic conditions." Moreover, this difference is not due to the formation of more higher carbohydrates in air, nor can it be explained by the slightly larger syntheses of fat and protein when oxygen is present.

Fidler (35) has provided similar evidence for the Bramley-Seedling and Sturmer apple and for South-African Navel Oranges. In his experiments, large samples of fruit were kept in air or nitrogen and transitional effects were ignored. Malic acid loss was the same in air and in nitrogen, but in all cases carbohydrate loss in air was significantly lower than that in nitrogen over periods up to 40 days. As the carbon loss as acid and carbohydrate was equal to the carbon of the end products, there is clear evidence of a P.E. in these tissues (see also 13).

Determination of the fermentation products.—Carbon loss in air or at the E.P. (if respiratory quotient equals one) is given by the OR; it may be calculated for anaerobiosis if carbon dioxide and alcohol (including acetaldehyde) are measured. Thus, Thomas (3) has shown that Boysen-Jensen's experiments provide clear evidence for a P.E. in some plants. If OR, NR, and ALC are measured in grams, it is easily shown that

$$x = \left[\left(1 + 1.91 \frac{\text{ALC}}{\text{NR}} \right) - \frac{\text{OR}}{\text{NR}} \right]$$

is positive if a P.E. exists. This fraction is positive in Boysen-Jensen's experiments with grapes, carrot roots, and pea cotyledons, plants in which the ratio ALC/NR approached the theoretical figure for alcoholic fermentation (0.81; 0.88; 0.65 as against 1.04). Negative or zero figures were obtained for potato tubers and *Tropaeolum* leaves; here the ratio ALC/NR was 0.1 to 0.5, and until we know what other substances are formed in F, these values for x do not preclude the existence of a P.E. in these tissues.

For the apple, Thomas & Fidler (12) have calculated carbon loss in nitrogen in two ways. For the Bramley seedling the fermentation is only 85 per cent alcoholic, the excess NR being labelled NR_{RES}. If this component of the NR is neglected, as possibly arising from nonmetabolic sources (15), the ratio carbon loss in nitrogen/carbon loss in air is 3.6; if the NR_{RES} is considered as coming from carbohydrate, the figure is 4.1. Thus, this variety of apple is shown to have a marked Pasteur effect by determination of alcohol and its accompanying NR only. The results may be stated in another way, that oxidation of one M.E. of hexose conserves 2.6 to 3.1 M.E. hexose from destruction. Similar unequivocal evidence for a P.E. has been obtained by Meeuse (7) and by Ranson (29); results are given in Table I.

In the above experiments the comparison was between air and nitrogen. Thomas and his co-workers (3), following Blackman's lead, have shown that

for some plants carbon loss is at a minimum at the E.P. The full extent of the P.E. is therefore only shown if the comparison is made of carbon loss in nitrogen and at the E.P. (Table I). Indeed a P.E. may not be revealed unless this is done, as seen in the figures for *Cucurbita*.

Measurement of the NR/OR ratio.—Most of the evidence for the existence of the P.E. in plants is indirect and is based on the ratio NR/OR obtained by gas analysis only. The classical paper on these lines is that of Blackman (4) who measured the time drift of NR and extrapolated the curve back to zero time in nitrogen. He was thus able to evaluate the ratio INR/OR where INR is the rate of carbon dioxide output at the moment of transition to nitrogen; in this way he hoped to avoid complications due to changing rates in air and nitrogen and the slow establishment of true anaerobiosis in a bulky tissue. For Bramley seedling apple fruits he obtained ratios varying from 1.3 to 1.5. Assuming $F=3$ INR, he thus obtained figures for carbon loss in nitrogen/carbon loss in air of about 4.0 to 4.5. This result may be expressed in another way—that the oxidation of 1 M.E. of hexose conserved 3.0 to 3.5 M.E. hexose from destruction. The figures are little different from those obtained by determining fermentation products (see Table I).

When the NR/OR or NR/OR_{E.P.} > 1 and provided that the NR arises metabolically, "it may be inferred, without further consideration of metabolic

TABLE II

DEMONSTRATION OF THE PASTEUR EFFECT BY USE OF THE RATIO NR/OR > 1

Plant and tissue	Ratio NR/OR		Type of F	Ref.
	Direct	Extrapolation	(—Unknown)	
<i>Pyrus malus</i> , fruits	>1	1.3 to 1.5	85 per cent alc.	Blackman (4)
<i>Prunus laurocerasus</i> , leaves		>1.0	—	Blackman (in 5)
<i>Daucus carota</i> , roots			70 to 100 per cent alc.	Choudhuri (26);
whole	1.4	1.4	70 to 100 per cent alc.	Boysen-Jensen (in 3)
sliced	>1.0	>1.0		Appleman & Brown (37)
				Turner (27); Marsh & Goddard (36)
<i>Citrus limonum</i> , fruits	>1.0		—	Biale & Young (24)
<i>Pastinaca sativa</i> , roots	>1.0		—	Appleman & Brown (37)
<i>Hordeum vulgare</i> , leaves	1.0		—	James & Hora (23)
<i>Oryza sativa</i> , seedlings	1.5 to 2.0		alc.	Taylor (31)
(M.Q.)				
<i>Cynara scolymus</i> , artichoke		1+	—	Stiles & Dent (38)
<i>Brassica</i> sp., mangold		1 to 1.2	—	Stiles & Dent (38)
<i>S. Lycopersicum</i> , tomato, fruits		>1.0	—	Gustafson (39)
<i>Vitis vinifera</i> , grapes	1.3 to 1.7		75 to 95 per cent alc.	Boysen-Jensen (in 3)
<i>Rhododendron</i> , leaves		1.5 to 2.0	60 per cent alc.	Ranson (29)
<i>Pisum sativum</i> , edible pea seedling		1.3 to 1.7	100 per cent alc.	Ranson (29)
<i>Solanum tuberosum</i> , tubers	>1		lactic+CO ₂	Boysen-Jensen (in 3) Boswell & Whiting (15)
<i>Mangifera indica</i> , mango		1.3	—	Singh (40)
<i>Psidium</i> sp., guava		1.3	—	Singh (40)
<i>Vicia faba</i> , germinating seeds	0.8 to 1.1		—	Wilson (41)

events" (3) that a P.E. exists. A summary of experimental results giving such high ratios is provided in Table II. The extent of the P.E. so demonstrated will be modified if $F < 3$ NR, if some of the NR comes from bound carbon dioxide (15), and if the extrapolation method of Blackman is used.

There is therefore good evidence for the existence of the P.E. in yeast and some 17 species of higher plants. In addition, there are a great many observations giving ratios NR/OR between 0.33 and 1.0. Details will not be quoted here as they cannot be properly interpreted until we have figures for alcohol production; moreover, only a few of these low ratios have been obtained by the extrapolation technique (25, 26, 42). We can only say that there is a strong possibility of the existence of a P.E. of small extent in many higher plants, apart from those named in Table II.

CHANGES IN THE EXTENT OF THE PASTEUR EFFECT IN ONE TISSUE

We have already seen that there are very few tissues in which the P.E. has been quantitatively evaluated and there are naturally few records which enable us to judge whether its extent alters with time, age, or under different environmental conditions. For carrot root slices, Turner (27) showed that, as the rate of respiration fell after slicing, OR and INR were correlated, but that OR fell more rapidly than INR; the ratio between them changed from about 0.7 to 1.8. Appleman & Brown (37) also record changes in the ratio with time of storage and with temperature. Thus, for tomato in the green stage the ratio was 1.7, in the red ripe stage it was 0.6. The most striking changes in this ratio were shown in potatoes where wounding or cold storage increased OR much more than NR and it is unfortunate that in this tissue the ratio has little meaning until we know more of the other fermentation products.

In contrast to the above, Blackman (4) showed that when the OR was considerably increased by raising the oxygen concentration above the E.P., the ratio INR/OR remained unchanged. He also showed that after a period in nitrogen, during which the NR rate fell to a low value, values for the ratio NR_{final}/IOR , obtained on return to air were similar to those obtained for INR/OR so long as reasonable assumptions were made concerning complications due to diffusion and "after effect." Thus Blackman considers that for apple the number of moles sugar conserved per mole oxidised remains constant over a wide range of oxygen concentration. Other plant tissues have not been investigated as fully as this, although Taylor (31) records a constant M.Q. for rice seedlings over the oxygen range from zero to E.P.

APPARENT ABSENCE OF THE PASTEUR EFFECT

Kostychev (41) recorded a number of examples in which the ratio NR/OR was 0.3 or lower and figures have been obtained more recently which are not markedly different from 0.3 (31, 37). As we have seen, we can place little reliance as yet on these values as estimates of the P.E. Stiles & Leach

(25), however, using Blackman's extrapolation technique obtained low ratios for a number of seedlings. They were somewhat above 0.3 for *Helianthus*, *Cucurbita*, and *Ricinus*, below 0.3 for *Zea* and *Lathyrus* while for *Fagopyrum* ratios of 0.36, 0.31, 0.36, 0.37 were obtained. In these experiments the NR changed only slowly after the initial sharp fall from the OR and the extrapolations appeared reliable. Ranson (29) has confirmed the value of 0.3 for *Fagopyrum*, but goes much further, measuring carbon loss by NR, OR, and alcohol determinations. In this way he has shown that carbon loss in this plant does not change when the oxygen concentration is raised from zero to the E.P. and actually increases (as it does for the apple) when the tissues are removed from the E.P. to air (Table I). This is the only recorded example of the absence of a P.E. determined by reliable methods. These results must be considered, however, in the light of Forward's work on barley (43), for which Barnell (44) also obtained a figure for INR/OR similar to that for the *Fagopyrum*.

The work of Ludwig *et al.* (45) must be dealt with separately. They conclude that in young roots and in legume nodules, oxygen may or may not conserve carbohydrate in one and the same tissue, according to the amount of intermediate, such as alcohol, present. However, although in separate experiments they provide evidence for the oxidation of added alcohol in these tissues, their figures for carbohydrate conservation are based solely on the rates of gaseous exchange: moreover, it is clear that higher oxygen concentrations increase OR (and therefore carbon loss) in these tissues and they have no data which allow comparison between carbon loss in nitrogen and at the E.P.

Like these roots, yeast also oxidises added alcohol rapidly, but this is not an argument for assuming it to be a normal respiratory substrate; the method of calculation used by Ludwig *et al.* (45) to estimate carbohydrate loss in air is not justified on the facts they present.

SO-CALLED INHIBITION OF THE PASTEUR EFFECT

It is possible to bring about aerobic fermentation (F_{O_2}) by lowering the OR, not only by reducing the oxygen concentration below the E.P., but also by adding an inhibitor of respiration such as cyanide. If then

$$NR_{O'_2} = 1/3(OR_{air} - OR_{O'_2}) \quad \text{or} \quad NR_{KCN} = 1/3(OR_{air} - OR_{KCN})$$

then no P.E. exists, except in the Burk sense. If, however, $NR_{O'_2}$ and NR_{KCN} are greater than the respective fractions, then a true P.E. exists and has been demonstrated by the use of cyanide. When this occurs (e.g., when cyanide reduces oxygen uptake but not carbon dioxide output) it is often stated that cyanide has "inhibited the Pasteur effect"; the reviewer finds this expression (containing a double negative) extremely confusing. The cyanide effect resembles that brought about by shortage of oxygen (which we do not regard as "inhibition of the P.E.") and there is no reason to suppose that it normally is due to anything but inhibition of OR.

The P.E. has been demonstrated in this way for *Lathyrus* seedlings, barley seedlings, apples, and carrot (12, 23, 36, 46) by the use of one or other of the respiratory inhibitors, cyanide, azide, and carbon monoxide. The most thorough work in this field is that of Thomas & Fidler (12) on apples. Although cyanide ultimately reduced the rate of NR in nitrogen, its effect in air was much more rapid and more pronounced; it was shown that inhibition by cyanide of the oxidation of one M.E. of hexose was accompanied by an increased hexose loss of 2.2 M.E. Assuming that cyanide itself does not change the glycolysis rate under these conditions, this gives a means of evaluating the normal P.E. and it is significant that the value obtained, 2.2, was very close to that measured for the same tissue by the ratio INR/OR, corrected by alcohol determinations. As far as the reviewer is aware, this is the only work on higher plants in which the values obtained by both methods have been compared, although Marsh and Goddard have calculated values for the M.Q. in cyanide (36).

While cyanide may be taken, therefore, as demonstrating rather than inhibiting the P.E., there are good reasons for regarding the effects of some inhibitors as modifying the extent of or destroying the P.E. Thus Warburg (47) has shown that carbon monoxide, while reducing Q_{O_2} , brings about a disproportionate increase in F_{O_2} , the M.Q. for normal tissue being different from that in the presence of the inhibitor. And again ethyl isocyanide may be properly said to inhibit the P.E. in animal tissues [Warburg (48)], for it raises the aerobic lactic acid production to the anaerobic level without affecting oxygen uptake. In an often quoted paper, Genevois (46) stated that he was able to find concentrations of cyanide which brought about aerobic fermentation (in *Lathyrus*) without affecting OR. He quoted only five short experiments and gave no details of the method of applying the cyanide in the two-vessel method of Warburg [see Krebs (49)]. Others who have experience with the use of cyanide would not feel able to rely on these results which in fact have never been confirmed. Marsh and Goddard could obtain no such effect with carrot tissue and Thomas & Fidler (12) could report no parallel for apples. However, an effect of the same kind can be produced with a different type of inhibitor, dinitrophenol (DNP). Thus, Newcomb (50) has shown that, in low concentrations, DNP reversibly stimulates respiration (in tobacco callus) while releasing aerobic fermentation. Higher concentrations of DNP inhibit respiration and allow increased F_{O_2} and with still higher concentrations both NR and OR are inhibited. The substance does not stimulate the rate of fermentation in nitrogen. Although Newcomb refers to the first result as inhibition of the P.E., he does not point out that his own data for the normal tissue used show only a small P.E. as measured by the NR/OR ratio. It therefore seems better to regard DNP as increasing carbon loss in air both by increasing OR and by releasing F_{O_2} . This fact must be taken into account in explaining the P.E.

EXPLANATIONS OF THE PASTEUR EFFECT

Two entirely opposed explanations of the P.E. have been strongly supported. A third, that of Lipmann (51), we shall discuss briefly. Lipmann considered that oxygen inhibited glycolysis and fermentation by reversibly inactivating one or more of the glycolytic enzymes. As Dixon (2) has already pointed out, it is difficult to explain on this hypothesis the effect of respiratory inhibitors. For plant tissue, Marsh & Goddard (36) showed that cyanide, carbon monoxide, and sodium azide all brought about aerobic fermentation in carrot root and they go on to say "Lipmann's theory is consistent with our results only if the direct inhibition of fermentation by oxygen is catalysed by cytochrome oxidase." The later theory of Lipmann (52) that the oxidised substance is coenzyme I is a different story which will be dealt with below.

The P.E. is usually regarded today as being due to either increased glycolysis in the absence of oxygen and of its accompanying respiration, or to oxidative anabolism accompanying respiration.

P.E. due to increased glycolysis in nitrogen.—With increased knowledge of the mechanism of glycolysis, it has been possible to suggest many likely ways in which a change from aerobic to anaerobic conditions might increase the rate of glycolysis. Two of these possibilities will be discussed here.

(i) *Pasteur effect and coenzyme I.*—In 1937, Dixon stated that "the oxidative removal of acetaldehyde," a substance necessary for the continuation of glycolysis, might be responsible for the P.E. and Ball (53) carried this suggestion further in 1939 by emphasising the role of coenzyme I in respiration and fermentation. He explained the suppression of fermentation (Burk's P.E.) as due to the oxidative removal of acetaldehyde and its consequent inability to return coenzyme I to the oxidised state. He does not seem to have gone further to use the same mechanism to explain the conservation of carbon by oxygen. Instead, he adopted an unsatisfactory teleological approach. It was left to Gottschalk (34) to propose that the P.E. of Dixon is in the last analysis "due to the circumstance that in the presence of oxygen part of the co-enzyme in the yeast cell is put out of action by persisting in the reduced state." In other words, oxygen acts by decreasing the rate of glycolysis.

Gottschalk first provides direct evidence (to be dealt with later) to show the absence of oxidative anabolism in yeast and goes on to provide biochemical evidence for his own hypothesis. His argument may be set out as follows:

(a) The rate of glycolysis in a yeast cell is limited by the amount of coenzyme I present. In the fermentation cycle, reduced coenzyme is formed and is in turn constantly reoxidized by the reaction with acetaldehyde, which is itself reduced to alcohol.

(b) In aerobic yeast the Krebs cycle does not operate [Krebs (54)] and acetaldehyde is the substrate for oxidation. Experimentally it is shown that the velocity of glucose oxidation by yeast equals that of acetaldehyde oxi-

dation when each substrate is separately added. The oxidation of acetaldehyde by molecular oxygen is supposed to take place (through the cooperation of a cyanide-sensitive alpha-dehydrogenase) with the formation of reduced coenzyme.

(c) In aerobic yeast, this reduced coenzyme, together with that produced in glycolysis, is oxidized by the diaphorase-cytochrome-oxidase-oxygen system. Experimentally it was found that even in the presence of oxygen, the reoxidation of Co I H_2 is effected by acetaldehyde if this is added and it is concluded that in yeast the velocity of the oxidation of Co I H_2 by the diaphorase system is lower than its oxidation by acetaldehyde. Therefore, under aerobic conditions, part of the reduced coenzyme persists in the reduced state and the rate of glycolysis falls. This accounts directly for the smaller carbon loss in air. Gottschalk notes that his hypothesis provides an explanation of certain facts not otherwise easy to interpret, for example, the disproportionate increase in NR_{O_2} when OR is partially suppressed by carbon monoxide [Warburg (47)] and variations in the M.Q. in different tissues. A lack of P.E. would be explicable as due to equality of the velocity of acetaldehyde oxidation under aerobic and anaerobic conditions, or to the presence of an excess of coenzyme in the tissue.

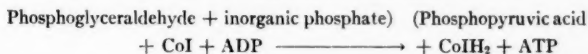
The application of Gottschalk's theory to the higher plant is attractive, but so far there is little evidence for it. The scheme would work equally well if in air the pyruvic acid were oxidized through the Krebs cycle; this would also provide excess reduced coenzyme to be removed oxidatively. There is already some evidence that a mechanism for such removal exists in the aerobic plant cell. Thus, James (6), working on barley, has provided evidence for a transfer of hydrogen through the system coenzyme I, dehydrogenase, ascorbic acid, ascorbic oxidase, oxygen. Waygood (55) has shown that, for wheat, ascorbic acid can replace methylene blue and function as a respiratory carrier mobilising the hydrogen from coenzyme I via an unknown pigment. Lockhart (56) and Davison (57) have obtained from pea seedlings a diaphorase which is concerned with the oxidation of reduced coenzyme and the oxidation of cytochrome-*c*, a system similar to that described by Gottschalk for yeast. It is evident that work on plant tissue is beginning to show that coenzyme I is important in higher plant respiration and that mechanisms exist for the oxidation of Co I H_2 , but we have no data on the rate of this oxidation in living tissue.

Although evidence is accumulating to indicate that a Krebs cycle, or something like it, operates in many plant tissues, it remains possible that, for some, acetaldehyde is the substrate of respiration. In the work on yeast it was possible to add acetaldehyde to aerobic cells lacking other substrates, but this cannot be done for tissues like the apple. There is surprisingly little known of the effect of acetaldehyde on plant respiration and we look forward to the full publication of Fidler's results. He has provisionally reported (58) that both apples and oranges take up acetaldehyde vapour which is, however, not accumulated; in nitrogen there is increased N.R. In oxygen the OR and

Q_{O_2} may be unaffected (apples) or stimulated (oranges) and F_{O_2} appears. Fidler considers it possible that in the presence of sugars, acetaldehyde is preferentially oxidized, some of the aerobic fermentation being derived from glycolysis products spared from oxidation.

It is clear that for higher plants we require more work on these lines and on the possibility of alcohol oxidation (45), and we need to know whether in fact glycolysis may be limited by coenzyme concentration.

(ii) *Pasteur effect and the phosphate cycle.*—In higher plants, as in yeast, phosphorylations play an important part in respiration and fermentation [James (6)]. Gottschalk has postulated that the concentration of coenzyme I may limit the rate of glycolysis; it will do so through the glycolytic step included in the partial equation of glycolysis in yeast:—



There may well be tissues or conditions in which the rate of glycolysis is limited not by coenzyme I but by the concentration of mineral phosphate or of adenosinediphosphate (ADP)—a substance which is also an acceptor of phosphate in a later glycolytic step. Like coenzyme I, its concentration in the cell is maintained by the operation of a cycle. Both R and F are supposed to lead to the temporary locking up of phosphate in adenosinetriphosphate (ATP), but whereas for each hexose molecule fermented there are formed 2 M ATP, some 36 to 50 M may well be formed during respiration. Release of phosphate with reformation of ADP is associated with endergonic synthetic reactions which will go on at different rates in air and nitrogen. If we assume that ADP may limit the glycolysis rate, then respiration may be regarded as a self-regulating process whose rate is automatically adjusted to the cell's functions. Rapid syntheses, for instance, would be associated with a rapid turnover of ATP and would maintain a high level of ADP and thus stimulate respiration itself. It is only a step from this to explain the P.E. in similar terms. Without attempting to assign priority we may mention the paper of Johnson (59) who provided indirect evidence (for animal tissue) to show that the P.E. was due to a reduction of glycolysis in air, brought about by the lowering in the concentration of phosphate acceptors as a consequence of the rapid esterification of phosphate in respiration.

At the present time there is no direct evidence that this hypothesis applies to plant tissue, although Bonner (60) has shown that added adenylic acid brings about an immediate and considerable stimulation of the rate of oxygen uptake in the *Avena* coleoptile. The work usually quoted (7) in favour of the "phosphate hypothesis" is that dealing with dinitrophenol, a substance shown to prevent the transfer of energy involved in the formation of ATP (61, 62). Robertson *et al.* (63) have recently shown that DNP in appropriate concentration stimulates oxygen uptake and stops ion accumulation by carrot tissue, while there are several records of DNP stopping protoplasmic streaming (64, 65). Its effect in releasing aerobic fermentation

has already been described (50). Sefts & Engelhardt (66) have gone further to show that a variety of substances (methylene blue, nitrite, DNP, azide), shown by analytical and tracer methods to interfere with phosphorylation, all brought about increased OR and released F_0 ("inhibited the P.E."). This work with substances added to animal tissues and yeast strongly supports the view that there is a link between respiratory phosphorylation and the P.E. We await biochemical demonstration with normal cells that the P.E. is associated causally with differences in the concentration of phosphate acceptors. Until this evidence becomes available, we are left in the position that on either hypothesis (i) or (ii) (pp. 157-58) there are strong reasons for thinking that on passage of a tissue from air to nitrogen there is likely to be an increase in the rate of glycolysis. We have no data which enable us to estimate the rate of this increase or of its final extent; hence, even if increased glycolysis in nitrogen accounts for part of the P.E. it does not necessarily account for it completely.

Pasteur effect and oxidative anabolism.—A theory which held the field for many years, originally propounded by Meyerhof (67), was that the P.E. was due to the existence of an anabolic reaction conserving carbon in air. Blackman called this process oxidative anabolism (OA) and he inferred it to be present after a very careful analysis of his experimental data for apples. Many workers, particularly in the animal field, have assumed that Blackman's work was a simple application of Meyerhof's, but this is far from true, and the classical paper of 1928 remains far and away the most careful and critical analysis of respiration data in the field of plant physiology. He is no doubt open to criticism in his assumption that glycolysis is not increased in nitrogen because it was at that time believed that oxygen did not decrease GL in yeast juice. But he was also driven to conclude, from the fall in NR in nitrogen and the rise in OR in oxygen above the E.P., that oxygen did have an indirect effect upon GL—that increased oxygen concentration actually increased GL. Such indirect effects were held to be relatively slow and his theory stands or falls on the hypothesis that, when a tissue is transferred from air to nitrogen, fermentation rates, corrected for diffusion effects in bulky tissues, can be measured before the decrease in the rate of glycolysis becomes appreciable. Arguing in this way, Blackman deduced that the only explanation of the very high initial fermentation rates as compared with the OR was that some of the products of glycolysis in air were anabolised back into the stream of substrates.

On this theory, on transfer from air to nitrogen, GL is at first unchanged and then falls with time; on the alternative view, transfer to nitrogen leads to a very rapid increase in GL which, however (explaining the data for apples), must soon reach a maximum rate and then decline. Admittedly "it is not yet clear whether the greater carbon loss is a factor causing this rapid decline of GL in nitrogen" (4). Even if this were so, the second effect of oxygen (increasing OR) has yet to be explained by those who regard oxygen as causing a decrease in glycolysis rate.

The concept of oxidative anabolism arose from a physiological approach; recent biochemical work in general is by no means opposed to it and "oxidative assimilation" in plant and animal cells is well established; this implies the synthesis of carbohydrates, proteins, fats, etc., in the nongreen cell, the energy coming (possibly via ATP) from oxidative processes. The question at issue is whether such processes are identical with Blackman's OA, that is, whether the products of glycolysis are used, in part, as the building stones for anabolism under aerobic conditions. It is well known that some of the acids arising in respiration may enter into the formation of amino acids, while Bennet-Clark & Bexon (70) have suggested a form of OA of carbohydrate from malic acid [see also Thomas & Beevers (71)]. Blackman's own views on the nature of the product of OA were that it might be a carbohydrate, or even malic acid, which would be further anabolised to carbohydrate or oxidised; "there is no need," he states "to regard OA as being necessarily an oxidised derivative of D; it might be reduced. A study of the actual oxygen consumption in all critical stages of apple respiration may help to throw some light on this uncertainty." More recently those discussing OA have suggested that its products might be not only carbohydrates but proteins. Forward (43) believes that indirect evidence is to be found in Barnell's observation (69) that (for barley seedlings in air) about twice as much hexose sugar went to the growth of dry matters as to respiration; . . . "since the proportion is the same as that between the hypothetical OA and OR (for these seedlings) the linkage of respiration and growth may be through OA. In young, actively developing seedlings in the dark . . . practically all of the anabolically consumed carbon would go into organised cell structure." Thus Forward tentatively proposes the identity of oxidative anabolism with oxidative assimilation. The application of this view to the resting tissue of the apple is more difficult, and Thomas (3) puts the matter as follows . . . "to prove experimentally that carbon is aerobically conserved because fats, amino acids et cetera are synthesised by OA at the expense of zymase cleavage products would be to achieve a major biochemical advance."

We now have one direct biochemical attack on this difficult problem. Gottschalk (34) has supplied what was sorely needed, a complete balance sheet for carbon metabolism in air and nitrogen. First it was shown, and confirmed in a subsequent paper (68), that when compressed yeast is suspended in phosphate buffer at pH 4.3 and supplied with glucose, fructose, or mannose, it ferments only 75 per cent of the total sugar consumed. About 50 per cent of the remaining sugar, consumed but not fermented, is synthesised, even under anaerobic conditions, to polysaccharides. The remainder (about 10 per cent of the total sugar consumed) is presumably utilised in the production of organic acids, fats, and proteins, for the yeast grows slowly even in pure nitrogen (Fig. 2). It would be of interest to know whether such anaerobic synthesis occurs in higher plants with their smaller fermentation rate.

When transferred to air or oxygen, the yeast cell, given glucose, continues

to ferment, but also carries out a small respiration. As Fig. 2 shows, the total carbon loss is reduced in air (Pasteur Effect) and the Meyerhof Quo-

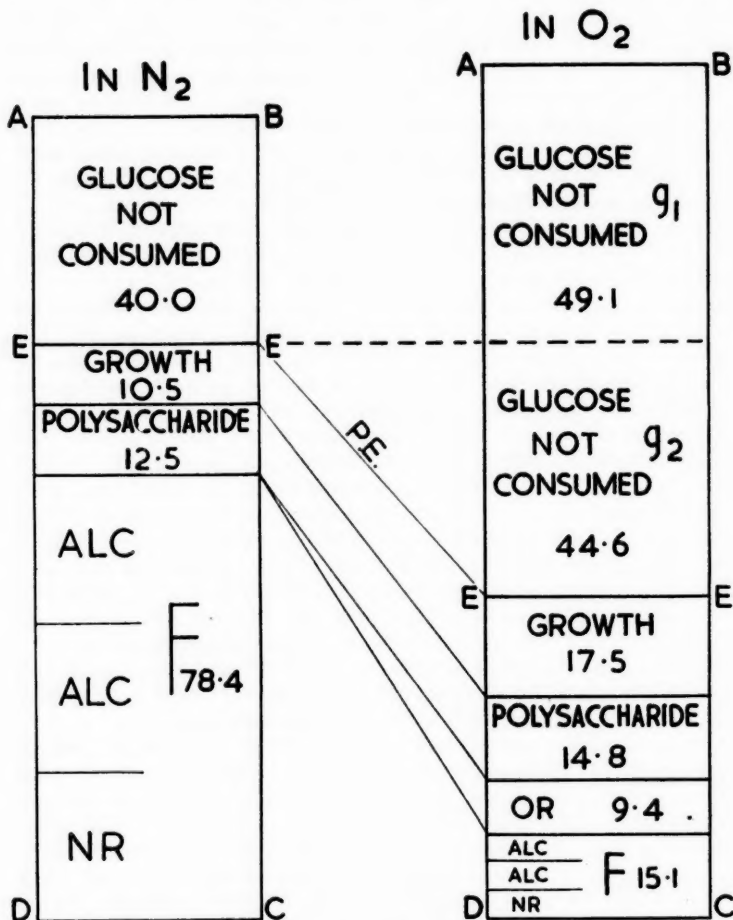


FIG. 2. Carbon balance sheet for yeast in air and nitrogen showing P.E. and absence of oxidative anabolism. Rectangles represent hexose equivalents. (Gottschalk's data)

tient is near two. The important discovery is that in air or in oxygen no more polysaccharides are synthesised than in nitrogen. Nor could there be found

any evidence for the more rapid oxidative synthesis of any other material at a rate sufficient to explain the P.E. It might, however, be argued that the P.E. is due to the oxidative anabolism of glucose itself, thus (Fig. 2), on the Meyerhof-Blackman view the fraction (G_2) would be considered to have been produced by glycolysis followed by oxidative resynthesis to glucose which would be found in the medium indistinguishable from that not consumed (G_1). Gottschalk considered this possibility in some detail and to test it he replaced the glucose by fructose and found that with this as substrate, a P.E. was also observed and all the nonconsumed sugar remained as fructose. The suggestion, he says, "that in the case of aerobic glucose fermentation, glucose is resynthesised, while in the case of aerobic fructose fermentation, fructose is built up, is not acceptable. A theory of Meyerhof that the P.E. in yeast cells is due to an oxidative resynthesis must accordingly be rejected." There appears to be only one flaw in the logic of his argument, for it is conceivable that when given glucose, yeast produces fructose by oxidative anabolism. This would not have been detected in this particular experiment. Gottschalk, however, considers this possibility extremely remote, as there is no evidence that yeast converts added glucose to fructose (private communication). We therefore accept Gottschalk's conclusions, but we need not conclude that because oxidative anabolism does not exist in yeast, the classical subject of Meyerhof's theory, that it exists in no higher plant. Moreover, it seems doubtful whether repetition of Gottschalk's experiment with higher plant tissue would provide us with an unequivocal answer as it does with yeast, owing to the well-known rapid interchange possible between the various sugars in such tissues. It seems probable, therefore, that the validity of the Blackman theory must be tested by further physiological work such as that of Forward (43) which is an extension of unpublished work with apples by Watson (72), in Blackman's laboratory.

Forward has come to the conclusion that it is not possible to rely on the ratio INR/OR as a test for the existence of the P.E.; that only a thorough survey of the effect of various oxygen concentrations upon the gas exchange will provide such a test; and that when it is applied in this way to seedlings, results very different from those reported by Leach (25) are obtained. Forward goes further than this in maintaining that her results showing the existence of a P.E. are explained most satisfactorily by assuming the existence of oxidative anabolism.

Forward deals with a population of growing seedlings and her analysis of the results is so complex and detailed that it is not possible here to do more than describe the key features. A set of uniform barley seedlings is germinated and allowed to grow in air, while the oxygen uptake and the carbon dioxide production are measured by continuous current method. The fermentation is known to be largely alcoholic (10, 11). The population is transferred to different gas mixtures at varied periods from the start of the experiment. The oxygen concentration available at the tissue surface is at any time a function of the applied external concentration, of the rate of passage

of the gas and the rate of oxygen uptake of the population; correspondingly, the effects of change from one gas to another are very varied. Forward distinguishes the following types of effect on lowering the oxygen concentration:—

(a) The primary effect, as when for a given rate of gas passage, the seedlings are transferred from air to five per cent oxygen. There is no F_{O_2} , OR falls and then continues to rise, as the seedlings grow, with a slope characteristic for that concentration.

(b) At concentrations lower than five per cent and for seedlings above a certain age the primary effect is succeeded by a secondary effect, a clear and sharp rise in OR and a concomitant development of F_{O_2} .

(c) At still lower concentrations of oxygen, OR falls and NR rises, but the rate of NR is modified by the existence of what are called excursions (tertiary effect) comparable with those observed by Leach (25) for seedlings in pure nitrogen and supposed to be due to the breakdown of some non-carbohydrate substrate.

The behaviour of the seedlings in 2.5 per cent oxygen provides a key to the analysis of the other effects which are quite inexplicable if taken singly. It is therefore necessary to deal with the ten experiments in 2.5 per cent oxygen in a little more detail. The effect of this gas concentration varies with the ontogenetic age of the seedling population, largely because this determines the internal oxygen concentration. Transfer of the seedlings from air to 2.5 per cent oxygen is critical when the transfer is made just as the seedlings pass out of the exponential phase of growth. Immediately on transfer, the OR falls (primary effect) to an approximately level phase of about 4 mg. per 5 gm. per hr., During this fall there is an outburst of NR. The NR falls to zero in a few hours while the OR continues at a steady rate (first adjusted state) for 20 to 60 hr. It then rises sharply (and the rise is shown not to be due directly to growth) to a new level (second adjusted state) of about 6 mg. per 5 gm. per hr. At the same time, aerobic fermentation again begins and reaches a steady state which is about $\frac{1}{3}$ OR.

These effects are diagrammatically summarised in Fig. 3. Forward explains them after making three assumptions:—(a) that OA exists and that, above the E.P., $OA = 2\ OR$; (b) that increased oxygen above the E.P. stimulates OR by increasing GL (the evidence for this is too complex to be set out here); (c) that for barley seedlings, as distinct from apples, changes in GL rate are rapid, a decreased rate equal to half that in air being accomplished 2 hrs. after transfer to nitrogen. This means that the ratio INR/OR_{AIR} cannot be used to measure the P.E. and that ratios of $\frac{1}{3}$ or less do not constitute proof that the P.E. is absent. The interpretation of such ratios "requires a knowledge of carbon expenditure throughout the range of oxygen concentrations" intermediate between zero and air.

The new scheme is summarised in Fig. 4A in which the line ADG represents carbon loss in different oxygen concentrations. Forward assumes that between zero oxygen and the E.P. (that is, while there is no surplus oxygen

in the tissue), GL remains constant and OR and OA both increase as F falls. Above the E.P., oxygen stimulates GL. The alternative hypothesis would assume that ADG represented both carbon loss and GL on which [if we adopt Forward's assumption (b) above] oxygen must have a dual effect. This is not out of the question, at least on the Gottschalk hypothesis, for the rate of oxidation of reduced coenzyme I might be a function of oxygen concentration. Forward bases her hypothesis partly on the improbability of such a dual effect and mainly on the experimental results with 2.5 per cent oxygen; in her view the shift to the second adjusted state is due to the re-

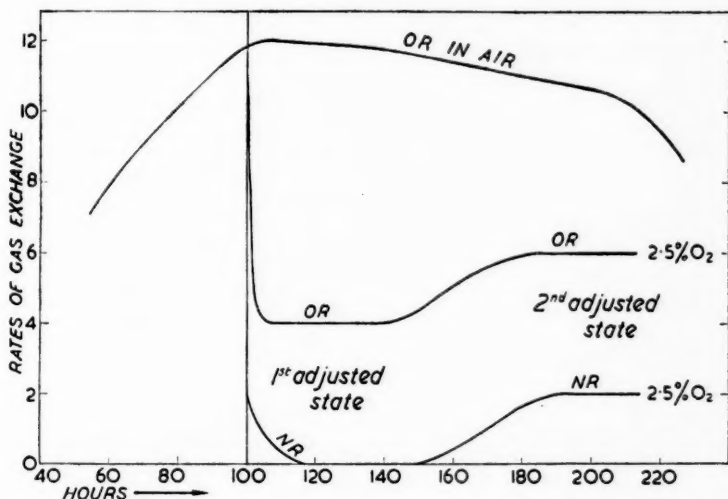


FIG. 3. Barley seedlings; OR and NR in air and 2.5 per cent oxygen plotted against time, showing spontaneous shift to second adjusted state due to falling internal oxygen concentration as seedlings slowly grow.

After Forward (43)

placement of OA by OR and NR as the effective internal oxygen concentration falls below the E.P. (Fig. 4B) and it must be admitted that a reversal in the effect of oxygen on GL (Fig. 4B, line CBD) is not likely to occur under just those circumstances which permit an increase both in OR and NR on passing below the E.P.

This analysis by Forward reopens the whole question of the existence of OA. Clearly her methods must be applied to other tissues and the fundamental assumption that the rate of OR above the E.P. is determined by glycolysis rate (rather than by oxidase activity or concentration) must be critically examined. It is likely that each tissue will provide its separate

if it exists at all, it is a function of the whole living cell and the study of it requires the analytical approach of Blackman equally with the application of experimental methods that have proved so useful in the elucidation of enzyme action.

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FORMATION, OCCURRENCE, AND INACTIVATION OF GROWTH SUBSTANCES¹

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INTRODUCTION

This review of the literature on formation, occurrence, and inactivation of growth substances of the auxin group is intended mainly to cover the period elapsed since the review by Zimmerman & Hitchcock in 1948 (190). Reference to earlier publications will be made occasionally in order to correlate them with recent studies. Other reviews dealing in part with this subject appeared during the period. Thimann (161) presented a much needed integration of older and recent work (more than 600 references). Chouard (27) gave an extensive review with approximately 1,000 classified references. Further reviews are by Bonner (15, pp. 442 to 456), Bünning (24, 25), Drawert (37), von Guttenberg (63, 64), van Overbeek (129), Sexton (147a, pp. 347 to 76) Sivori (148), Thimann (162), and Weevers (173).

The term auxin will be used in the wider sense (151). The abbreviation IAA stands for indoleacetic acid.

FORMS OF AUXIN AND AUXIN-PRECURSORS AND METHODS FOR THEIR EXTRACTION AND DETERMINATION

The forms in which auxin and its precursors may occur in plants may be roughly classified as follows: (a) free acids; (b) IAA-protein complexes; (c) tryptophan-containing proteins; (d) low-molecular precursor-complex in endosperms; and (e) neutral forms [for a detailed discussion of these forms see previous reviews (15, 128, 129, 151, 162, 190)]. In addition to these groups the inhibitor-auxin complex studied by Söding and his co-workers will have to be considered. (The existence of still another type of bound auxin was postulated by Bouillenne & Bouillenne-Walrand (17, 18), namely a complex of rhizocaline, auxin, and a specific enzyme. Galston & Hand (51) suggest the possibility that calines are identical with adenine.) It is necessary to subject a given sample (or parallel samples) of plant material to several kinds of extraction, and in some cases more than one type of test, in order to get a complete picture of the actual and potential content of auxin in the sample.

Free acids.—Methods for extraction of the free auxin actually present in the sample at a given moment should be designed to prevent a chemical or enzymatic release of bound auxin during the period of extraction. It has been proposed to eliminate enzymatic release of auxin by means of boiling (59) and by using short periods of extraction (1½ hr.) (131, 133). Wildman &

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Muir (185) suppressed the enzymatic release by extracting at 0°C. or by adding cyanide. At 0°C. the yield of auxin from tobacco ovaries was only insignificantly higher after 16 hr. of extraction than after 1 hr., whereas at 23°C. the yield increased four to five-fold from the 1st to the 16th hr. Extractions for one to two hr. at either temperature yielded almost identical quantities of auxin, which may be assumed to represent the free auxin present. Cyanide did decrease the auxin yield at 23°C. (16 hr.), but also interfered with the *Avena* test. It may be possible to develop methods to remove quantitatively the cyanide prior to the test or to use other inhibitors. As long as this has not been done, however, extraction at room temperature for 1 to 2 hr. or at 0°C. for 1 to 8 hr. seems to be the most satisfactory method for extraction of free auxin. Boiling of plant material may either release bound auxin chemically or liberate growth inhibitors (131).

Bound auxin in general.—"Bound auxin" is frequently used as a practical term for auxin detectable only after autolysis or treatment of plant material with enzymes or chemicals. In some cases it is undetermined whether the auxin formed by such treatments was actually bound or just present as a free precursor-molecule which needed only a minor change to become active. Further, it seems difficult at present to distinguish clearly between IAA-protein complexes and tryptophan-containing proteins. Tryptophan yields small amounts of IAA under various conditions such as alkaline reaction or ultra-violet irradiation (11).

Wildman *et al.* (180, 182, 183) on the basis of extensive investigations on spinach leaf proteins [see previous reviews (151, 190)] arrive at the conclusion that IAA as such combines with a specific protein in leaves to form a phosphatase which is assumed to play a role in the transfer of energy required for growth. This view is in accord with the observation (16) that soaking of *Avena* coleoptiles in an IAA solution increases the phosphatase activity and the content of bound auxin in protein prepared from the coleoptiles. The existence of an IAA-protein was questioned by Schocken [(146); see also (190)] who suggests that the auxin obtained from the phosphatase-protein (180) may have arisen from tryptophan rather than from preformed IAA bound to the protein. Schocken's results do not exclude the possibility of the existence of an IAA-protein complex, which on theoretical grounds is highly probable. General acceptance of the idea, however, that the above-mentioned phosphatase is the functional form of auxin must await further evidence.

The simplest way to liberate bound auxin is to extend the extraction with "wet ether" over a prolonged period. Under such conditions enzymes present in the plant material will release the auxin. In dried *Lemna* powder (164, 165) 12 or more weeks are required to exhaust the plant material of auxin. In pineapple leaves (133) periods of about two weeks seem to suffice. Gordon & Nieva (56), in their studies on the release of bound auxin in pineapple leaf breis, added cyanide (0.005 *M*) in order to prevent enzymatic inactivation of the IAA formed. They found no increase in the yield of auxin after four days of extraction.

Addition of enzymes for the release of bound auxin from plant materials

was originally introduced by Skoog *et al.* (152, 165). Of the proteolytic enzymes, chymotrypsin was reported to be more efficient than trypsin. Wildman & Bonner (180), working with spinach leaf proteins, found, however, that trypsin gave almost as high auxin yields as alkaline hydrolysis when the trypsin treatment was preceded by a treatment with pepsin. Moewus (115, 119) and Muir (120) used pancreatin for the release of auxin from a bound state. All of the enzyme preparations mentioned would have the capacity for attacking proteins and thereby possibly releasing auxin from an auxin-protein complex, or they might release tryptophan, a small amount of which might in turn become converted to IAA. Commercial pancreatin, however, might also release auxin if it were bound to some complex by an ester bond.

In this connection it is worth mentioning that Kulescha (87) found that agar, gelatin, and gum arabic, none of which contain tryptophan, yielded considerable quantities of auxin upon treatment with trypsin. The possibility that this auxin came from the trypsin preparation [chymotrypsin (146) yields auxin upon alkaline hydrolysis] is eliminated by the fact that cotton, paper, and wood yielded no auxin by a similar treatment. "Bound" auxin apparently is present in several organic materials.

Inhibitor-auxin complex and low-molecular precursor.—The existence of a growth substance or precursor possessing the remarkable property of being resistant to treatment with hydrogen peroxide has been reported by several German workers (42, 43, 111, 170). This substance is regarded as a growth inhibitor capable of releasing auxin *in vivo*. The substance, originally termed "inactive growth substance" by Voss (170) was renamed "antiauxin" by Funke & Söding (43). The reviewer would prefer a term like inhibitor-auxin complex as being more descriptive. "Antiauxin" may then be reserved as a term for substances which inhibit growth by interfering or competing with auxin. The non-auxin component of the inhibitor-auxin complex would be an antiauxin. The presence of the complex was demonstrated in seedlings of *Zea mays* and *Avena sativa* and in tubers of *Solanum tuberosum*. It was obtained (a) by diffusion from tissues to agar or filter paper, (b) by diffusion from breis to agar, and (c) by extraction of breis with water or alcohol and placing drops of the extract on agar blocks to be tested.

The inhibitor-auxin complex has no effect in Söding's day-light *Avena* test. It does, however, produce curvatures in excised coleoptiles cut 24 hr. before the test ["Koleoptilentest" (41)]. The test used most extensively was the "Zylindertest" (42, 43, 170). In this test the straight growth of coleoptile sections supplied with agar blocks containing the extract is compared with the growth of control sections to which plain agar was applied. The effect of the extract is studied both in freshly cut sections and in sections cut 24 hr. before the test. If a brei of potato tubers is treated with hydrogen peroxide and tested on "old" sections, these respond to the extract by showing a growth increase within 2.5 hr. They continue to grow faster than the controls throughout the test (23 hr). Similarly treated but freshly cut sections, on the other hand, grow slower than the controls for at least 5 hr. Later they

start growing faster, and by the end of the test they too, are longer than the controls. The different response of the two kinds of coleoptile sections to the same extract is interpreted as follows. The brei extract, after treatment with hydrogen peroxide contains no free auxin, only the inhibitor-auxin complex. The complex inhibits growth in the freshly cut coleoptile sections, which are unable to release auxin from it. Later, when a "physiological tip" has developed, the upper part of the section becomes capable of releasing auxin from the complex. This auxin makes the section grow faster than the control. In the old sections, on the other hand, the regeneration of the physiological tip has already taken place. They start immediately to release auxin from the complex. The auxin released not only counter-balances the inhibitory effect of the remaining portion of the inhibitor-auxin complex, but actually stimulates growth to a rate higher than that of the controls.

In addition to its stability to hydrogen peroxide, the inhibitor-auxin complex is reported to possess the following properties (43): (a) sensitivity to hot hydrochloric acid; (b) stability to hot ammonium hydroxide; (c) its molecular weight is higher; and (d) its solubility in water is lower than that of IAA (or auxin *a*). It is, furthermore, capable of both acro- and basipetal transportation in coleoptiles.

In the reviewer's opinion, the inhibitor-auxin complex might well be identical with the low-molecular weight auxin precursor studied by Avery and his co-workers (3, 9, 10). This possibility was not considered by Funke and Söding (43) evidently, because their manuscript, although not printed until 1948, was finished as early as May 1944. There are a number of similarities between the substances studied by the two groups of workers: (a) both substances occur in storage organs (endosperms, potato tubers); (b) both have a molecular weight higher than that of IAA, but do not seem to be protein complexes; (c) the water solubility of both seems to be lower than that of IAA; and (d) both are resistant to boiling with hot alkali.

Other points will have to be clarified by further experimentation. In an experiment reported by the American workers (10), 40 per cent of the potential precursor activity was destroyed by hydrogen peroxide. Whether the inhibitor-auxin complex of the German workers is actually 100 per cent stable to this reagent can not be seen from their data. Von Guttenberg & Lehle-Joerges (67) report that they were unable to demonstrate the presence in grain extracts of a growth substance stable to hydrogen peroxide. These workers, however, used only the standard Went technique and not the isolated coleoptile sections in their bio-assays. Because of the low solubility of the precursor at low pH values, the American workers do not give information on the stability of the precursor to acids. A point difficult to reconcile with the assumption of identity of the two precursor complexes is the fact that the inhibitor-auxin complex, although stable to boiling with ammonium hydroxide, failed to yield free auxin upon treatment with the base (43). No tests with stronger, non-volatile bases were reported.

Von-Guttenberg & Lehle-Joerges (67) suggest that the auxin released from the precursor studied by Avery and his co-workers (3) may be an anti-

fact since it was obtained by treating ground, whole grains, rather than extracts, with base. This criticism does not apply to all of the experiments in the paper cited and certainly not to the later work of Berger & Avery (9, 10). The failure of von Guttenberg & Lehle-Joerges (67) to demonstrate any increase in auxin activity by treating either ground, whole grains or grain extracts with base may be easily explained by the enormous concentrations at which their extracts were tested. When the extract of 100 gm. of maize kernels is concentrated in 1 ml. of agar, *Avena* test plants will not increase their curvatures if the auxin concentration is raised.

The properties reported for the inhibitor-auxin complex seem to be in accord with the assumption that IAA is the auxin present therein. Yet, the authors (43) reject this possibility on the basis of the stability of the complex to hydrogen peroxide. The reviewer, however, in agreement with von Guttenberg (63), considers it very likely that the auxin component of the complex is IAA or a compound related to IAA. Funke & Söding themselves show that pure IAA is only partially destroyed by their method of treatment with hydrogen peroxide. If, in future experiments, the complex should actually show a higher degree of stability to hydrogen peroxide than does pure IAA, there is still the possibility that IAA (or its derivative) is protected against the action of this reagent when in combination with the other component of the complex. The properties of the free auxin released from the complex were not investigated.

Neutral growth substance.—When an ether extract containing free auxin or auxin released from a bound state has been obtained, different workers proceed in different ways to prepare this extract for biological assay. Some (59, 131, 133, 152, 164, 165, 185) reduce the ether in volume and take it up in molten agar to be tested on *Avena* coleoptiles. Others (20, 53, 55, 56, 100, 172) subject the extract to partition between ether and water at different pH values in order to separate neutral and acid growth substance. The neutral growth substance (presumably indoleacetaldehyde) is considered an immediate precursor of IAA (see later) but it does give curvatures in the *Avena* test because it is rapidly converted to IAA in the coleoptile tissue (98). The capacity for such conversion may vary from one kind of test object to another, independently of the capacity for responding to IAA. Therefore, a separation of the neutral and acid growth substances should always be made before auxin assay. This should be done also in cases in which the crude extract is a solution in alcohol (103) or in water (115, 119), and also in experiments on the enzymatic formation of auxins from other compounds.

Inhibitors.—It has been shown in several instances (20, 40, 53, 94, 100, 102, 103, 116, 133, 136) that plant extracts may contain substances which retard growth and counteract the effect of auxins in various biological tests. It is entirely possible that such substances in the living plant have the function of balancing auxin activity. It is also possible, however, that they are, to some extent at least, artifacts, formed during the extraction. In either case it would be very desirable to separate them from the auxins and

auxin precursors and to determine the activity of each group independently. Methods for such separations are few and have not been used extensively. Chemical methods are difficult to apply to this problem, because the chemical nature of the inhibitors is largely unknown. A partial separation was attained by Boysen-Jensen (20), Goodwin (53), and others using a physical method based on the differential diffusion velocity of auxins and inhibitors in agar. Differential adsorption techniques might prove useful for such separations.

Once the inhibitors have been separated from the auxin, methods are available for determining their biological activity. Such methods have been based on the determination of the retardation of growth of intact coleoptiles (94, 102, 103) or decapitated coleoptiles (43, 94, 100, 154) and retardation of root growth (2, 80, 113, 114, 115, 117, 119).

Biological tests.—Several test methods developed mainly to determine the effects of synthetic substances, generally available in relatively large quantities, have been described recently. This section, however, will deal mainly with test methods which seem applicable to plant extracts. The tests used for the study of the inhibitor-auxin complex (42, 43, 111) were described above.

Muhle Larsen (92) analyzed the genetic purity of Victory oats (Svalöv strain, cultivated in Denmark) with respect to its sensitivity to IAA. In a series of *Avena* tests individual plants showing low, medium, and high sensitivity to given concentrations of IAA were selected and grown to maturity in the field. Individuals which had shown low sensitivity to IAA yielded a progeny with a significantly lower sensitivity than that of progeny of highly sensitive parents. The variability within these two groups, as well as in the intermediate group, was lower than in the original population. Thus it is possible within the otherwise pure strain of Victory oats to select for higher auxin sensitivity and lower variability in the *Avena* test. [See also the work of Judkins (79) reviewed by Skoog (151).]

Bentley (8) on the basis of an analysis of the coleoptile section test recommends certain procedures to reduce its variability. Adding sugar to the test solution is not recommended, but floating the sections is. According to Bonner (14), the addition of sugar, arginine, and manganese increases the response of isolated coleoptile sections to IAA. Rietsema (141, 142) recommended soaking the sections for 6 hr. before the test.

Kent & Gortner (83) greatly improved the reproducibility of the pea test by subjecting the etiolated seedlings to 10 foot candles of red light for 4 hr., starting 32 hr. before harvesting. Young cucumber seedlings (28) and tomato ovaries (105) were found to be sensitive test objects for phytohormones in plant extracts.

The root test developed by Moewus (113, 114, 117, 118) is of particular interest because it makes possible a simultaneous determination of growth-promoting and growth-retarding activity. Moewus uses the roots of rigidly selected, intact seedlings of garden cress (*Lepidium sativum*) as test objects. The content in a given plant extract of IAA is determined by its promoting

effect on root growth at sufficient dilution. Higher concentrations of the extract exert a retarding effect. The degree of retardation caused by IAA in the extract is known from experiments with pure IAA. Any further retardation is ascribed to inhibitors in the extract and may be expressed in terms of biologically equivalent amounts of coumarin. This growth inhibitor is required in concentrations of about 100 times that of IAA to give a 50 per cent retardation of root growth in the cress test. Coumarin does not have any promoting effect in any concentration in Moewus' test. Therefore, any promotion of growth by the extract is taken as evidence that the extract has been diluted enough to get the concentration of IAA down into the range where it has a promoting effect and to bring the concentration of inhibitor down to the range where it has no effect at all. The basis for this procedure is the assumption that the growth-retarding activity of any inhibitor in the extract is lower per unit weight than that of IAA. Linser (104) was able to show that certain concentrations of phenoxyacetic acid and 2,4-D have a promoting effect on root growth in the cress test. In Moewus' test, 5 ml. of extract are needed for each concentration to be tested. A similar test requiring only 0.1 ml. (in the form of an agar square weighing 100 mg.) was developed by Ashby (1), using the tiny seedlings of *Artemisia absinthium*. The roots of this plant and of cress were stimulated in their growth by IAA in concentrations from 0.1 to 0.001 $\mu\text{g. per l.}$ They showed 50 per cent inhibition at 16 $\mu\text{g. per l.}$ (1, 117, 118) or 35 $\mu\text{g. per l.}$ (104).

CHEMICAL IDENTITY OF NATURALLY OCCURRING GROWTH SUBSTANCES

That IAA might occur in a higher plant (*Celtis reticulosa* Miguel) was suggested (although not conclusively demonstrated) as early as 1909 by Herter (76). The experimental evidence for the normal occurrence and hormonal function of IAA in higher plants [see (151) and (190)] is still augmenting. As means for indirect identification of growth substances, determinations of their molecular weight (from their diffusion velocity in agar) and of their sensitivity to boiling with acid and base are frequently used. Previous summaries of diffusion experiments (86, 96) may be supplemented with the results given in Table I, which shows that potato tubers, tomato stem tips, pineapple leaves, coleoptile tips, sugarcane nodes and the fungus *Phycomyces* contain a growth substance which resembles IAA with respect to its diffusion velocity.

Kramer & Went (86) found no difference in molecular weight of auxin extracted with ether from tomato stem tips and auxin diffused into agar from the tips. In agreement with several earlier reports Wildman & Bonner (181) in their first experiments with auxin diffused from excised *Avena* coleoptile tips found values corresponding to a molecular weight of 306, indicative of auxin *a* or *b*. They discovered, however, that if auxin diffused from the tips is extracted from the receiving agar blocks with ether and again transferred to agar, the diffusion coefficient is then close to that expected for IAA. The ether extraction and the transfer of the extract to agar are reported to

be attended by a negligible loss in auxin activity. Wildman & Bonner apparently used the four middle agar disks of a stack of six for their calculation of the diffusion coefficient. Kawalki's table (82), however, is not directly applicable to such conditions. The diffusion coefficients reported (temperature?) are probably at least 10 per cent too low, but of course the similarity

TABLE I
RECENT ACID-SENSITIVITY TESTS AND DIFFUSION EXPERIMENTS
WITH GROWTH SUBSTANCES

<i>Material</i>	<i>Sensitivity to acid*</i>	<i>Molecular weight found</i>	<i>No. of exp. with plant material</i>	<i>No. of exp. with pure IAA (M=175)</i>	<i>Ref.</i>
Potato tubers: acid fraction	labile	186	23	10	(72)
Tomato stem tips: ether extract	labile	203	11	2	(86)
diffusate		201	12		(86)
Pineapple leaves: acid fraction	labile	181	16	13	(55)
<i>Avena</i> coleoptiles: crude diffusate		306	several		(181)
purified diffusate		206	2	2	(181)
Sugarcane, nodes: ether extract	labile	†			(132)
<i>Phycomyces</i> : acid-labile fract.	labile	167	3	2	(169)
acid-stable fract.	stable	330	5	4	(169)

* Acid-labile materials were alkali-stable and vice versa.

† Diffusion velocity stated to be similar to that of IAA rather than auxin *a*. No actual figures given.

in behavior of two substances can be shown as clearly by the method of Wildman & Bonner as by the usual method involving a total of four disks.

In most of the experiments listed in Table I, the identity with IAA of auxin showing a low molecular weight was further indicated by its sensitivity to acid. Acid-sensitive auxin has been obtained in several other cases: sugarcane (131, 132); bean hypocotyls (131); corn coleoptiles (132); and *Chorella* (143). Additional examples will be given below.

Wildman & Bonner (181) undertook the task of extracting 23,000 *Avena*

coleoptile tips with ether. They subjected one-half of the concentrated extract to a Salkowski test which clearly showed the presence of IAA in the extract. The amount of IAA corresponded to 80 per cent of the auxin activity obtained in a parallel bio-assay. Whether the difference of 20 per cent is due to experimental errors or to the presence in the extract of an auxin which is not IAA is unknown. A similar set of experiments with an auxin solution obtained by letting the active material diffuse from approximately 20,000 coleoptile tips to water did not yield as clear-cut results. A slightly pink color was obtained in the Salkowski test, but a turbidity of the diffusate interfered with the test. It is still possible that IAA is only one component of the diffusible auxin in coleoptiles.

The demonstration of the presence of IAA in coleoptiles (132, 181) is in disagreement with the results of von Guttenberg & Lehle-Joerges (67) who found no alkali-stable auxin in coleoptiles of *Avena* and *Zea*. They did, however, find alkali-stable auxin, presumably IAA, in the first internode and scutellum of maize seedlings as well as in the scutellum of *Avena* and in entire caryopses of maize and wheat. An alkali-stable auxin was also present in seeds of *Tropaeolum* and buckwheat and in buds of *Syringa*. Meyer (111) confirmed the observation by Hemberg (72) that an acid-labile auxin (presumably IAA) is present in potato tubers.

The accumulation of evidence for the occurrence of IAA in plants and the relative scarcity of results indicating the presence of auxins *a* or *b* have created some doubt about the existence of the latter auxins (189). There are, however, several observations, also of recent date, which indicate the presence in plants of a growth substance which may be auxin *a*. If these observations are not accepted as evidence for the existence of auxin *a*, they will have to be fitted into the hypothesis that IAA is the only natural auxin by *ad hoc* assumptions which may then be tested in the future.

Evidence for the occurrence in plants of auxin *a* (aside from its early isolation from plant materials) was seen in the diffusion experiments which yielded molecular weights of about 300 for natural auxins. This evidence has been weakened by the finding by Wildman & Bonner (181) that diffusible auxin after ether extraction yields values closer to 175 (IAA), while the unpurified diffusate yields values about 300 ($M = 328$ for auxin *a*).

Verkaaik (169) confirmed earlier work on *Phycomyces* by finding a low molecular weight for the auxin present in that fungus (Table I). This auxin was destroyed by acid but not by alkali. After treatment of the *Phycomyces* extract with acid, however, there was still some auxin activity (about 5 per cent) left which was not destroyed by repeated boiling with acid (3 hr.) but disappeared by boiling with alkali. Diffusion experiments with the acid-stable auxin showed a molecular weight of 330 for this substance. These results indicate the presence of auxin *a* in *Phycomyces*. The acid-stable auxin was found only in young sporangiophores possessing phototropic reactivity, but not in old ones lacking this property. Verkaaik also found that acid-treated *Phycomyces* extract, but not IAA, was capable of restoring the phototropic sensitivity in deseeded, double-decapitated *Avena* coleoptiles. This

effect he takes as further evidence for the identity of the acid-stable auxin with auxin *a*. The effect observed, however, might also have been caused by some photoreceptor present in the *Phycomyces* extract.

Verkaarik's is the first report on the finding of an acid-stable auxin in fungi. There are several recent findings of acid-stable (and alkali-labile) auxin in materials prepared from green plants. Gordon (54), working with wheat grains, found an acid-stable auxin as a component of the free auxin of the grain, the remaining free auxin being of the IAA-type. Concomitant with the IAA-type, acid-stable auxin was also present in a bound or adsorbed form in several well-characterized proteins prepared from the grains. Avery *et al.* (4) reported the presence of large quantities of an alkali-labile (acid-stable?) auxin in wheat kernels. Roborgh & Thomas (143) found 75 per cent of the auxin extractable from *Chlorella* to be acid-stable.

Several German workers (30, 42, 43, 67) and Hemberg (72, p. 202) found acid-resistant auxin in various plant extracts. Von Guttenberg (62 to 65) holds the view that the action of IAA as a growth regulator is indirect, IAA just provoking the liberation or formation of auxin *a*. This view is based in part on experiments (30) which showed the presence of large amounts of acid-stable auxin in plants treated with IAA. Information about the normally occurring quantities of acid-stable auxin, however, seems to be lacking. Furthermore, only in a few cases were the extracts tested at more than one concentration. The quantitative relationships, therefore, are difficult to evaluate. In interpreting some of the above-mentioned results it should be borne in mind that auxin has in some cases (6, 111, 120, 166) been reported to be released from a bound state by hydrolysis with acid. Whether this auxin is actually acid-stable, or whether it is IAA liberated in quantities large enough to leave some undestroyed by the acid, was left undetermined. In one case (6) acid-released auxin was shown to be alkali-stable (IAA?). Several cases of a truly acid-stable auxin obtained from plant material, however, still remain, and a central point in the problem of the existence of auxin *a* is the question: if not auxin *a*, what then is the acid-stable auxin in plants?

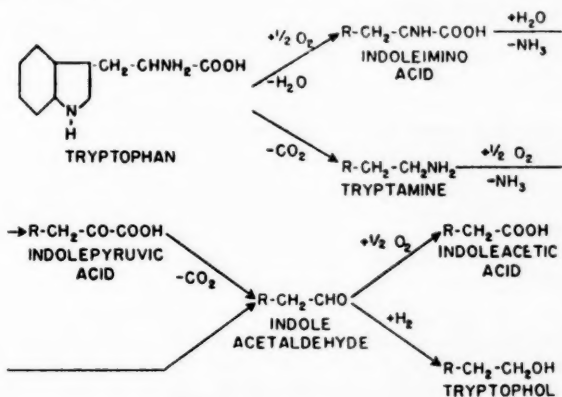
Reports [such as (169) and others] of a differential response of test plants to pure IAA and to plant extracts (presumably containing auxin *a*) constitute another group of observations which will have to be reinterpreted if auxin *a* is to be rejected as a naturally occurring and functioning growth hormone. A certain kind of differential response is discussed by von Guttenberg (63, 64), mainly on the basis of previous experiments (65) with *Avena* coleoptiles and *Helianthus* hypocotyls which were made auxin-free or at least very low in auxin. Unilateral application of diffusates from coleoptile tips and extracts of cabbage leaves to such organs resulted in curvatures which started about 1 hr. after the application and reached their maximum in about 2 hr. IAA, on the other hand, produced curvatures which did not start until 3 hr. (*Avena*) or 10 hr. (*Helianthus*) after the application and required 7 or 16 additional hours, respectively, to reach their maximum. Normal plants, which were decapitated, but not made auxin-free, showed

essentially identical responses to IAA and to plant extracts. The IAA concentrations applied seem to be extremely high (1 to 100 mg. per l.). The authors conclude from these and other results that IAA is not directly active as a growth promotor, but liberates auxin *a* or starts its production from some precursor [an idea which Ruge (144) correlates with the suggested function of IAA in respiration]. The correctness of this interpretation will depend on whether or not the plant extracts and diffusates actually contained auxin *a*. For the *Avena* coleoptile this question is not settled (67 versus 181). The cabbage extract used may have contained largely indole-acetaldehyde (96). Leaving open the question whether auxin *a* was also present in the preparations and was responsible for the von Guttenberg effect (the quick response of the test plants to plant extracts as opposed to the delayed response to pure IAA), other explanations are also possible. The extracts and diffusates are far from pure, and substances other than auxin (auxin *a* or IAA), which may be necessary for renewed growth in the starved plant organs, may be responsible for the observed effect. Von Guttenberg maintains that IAA moves non-polarly. This point needs to be reinvestigated, particularly at lower concentrations of IAA.

In summary it may be said that IAA is well established as a naturally occurring auxin. The hypothesis that IAA is the only auxin in plants, however, is at present unable to offer a satisfactory explanation of certain observations, particularly of the occurrence of an acid-stable auxin and of the von Guttenberg effect.

FORMATION OF GROWTH SUBSTANCES

Several plant tissues have the capacity to form IAA from tryptophan via intermediates, the nature of which has not yet been determined with certainty. A scheme showing possible steps in this process is presented below. The evidence for the various steps in the conversion will be discussed in the following paragraphs.



Conversion of tryptophan to indoleacetic acid. The over-all reaction.—Thimann (160) showed that the amount of IAA produced by the fungus *Rhizopus* was determined by the amount of tryptophan present in the substrate. Boysen-Jensen (19), on the other hand, who compared the value of 12 amino acids for the formation of auxin by *Aspergillus niger*, found histidine, leucine, lysine, phenylalanine, proline, and tyrosine to be superior to tryptophan in this respect. It may be assumed that the fungus is capable of forming tryptophan from various amino acids.

The capability of living tissues to form auxin from tryptophan has been demonstrated for spinach, pineapple, pea, and tomato (normal and zinc-deficient) by Wildman *et al.* (184), Gordon & Nieva (56), Galston (45), and Tsui (166), respectively. Gustafson's results (61) also indicate a conversion of tryptophan to auxin in tomato shoots. The tryptophan-converting capacity was found in corn endosperm (153), cell-free preparations from spinach leaves (184), etiolated *Avena* coleoptiles (181), pineapple leaves (56), and crown-gall and callus tissues of sunflower (75). The tryptophan-converting activity in lyophilized tobacco ovaries is exceptionally high (185). Gordon & Nieva (56), working with the etiolated bases of pineapple leaves, showed that the sensitivity to hot acid and base of the auxin formed from tryptophan was similar to that of synthetic IAA. Although actual identification of the auxin formed in the above and other similar experiments with higher plants (149) is lacking, it must be considered very highly probable that this auxin is IAA. Since good sources of the conversion enzymes are now available from higher plants it might be possible to isolate the active conversion product from such material or to produce enough of the auxin for at least a colorimetric identification, using a modified Salkowski test (57a, 155).

Interesting information on the occurrence of the enzyme system which converts tryptophan to auxin was obtained by Wildman & Bonner (181). They found that the auxin-forming activity was much higher in the topmost 5 mm. of the *Avena* coleoptile than in the 5 mm. immediately below and decreased further toward the base of the coleoptile. Three hours after decapitation the subterminal zone showed an increased capacity for enzymatic auxin formation. These findings are in accord with the known capacities of normal tips and regenerated "tips" to produce auxin. As the authors point out, however, it is undetermined whether the increased yield of auxin from regenerated "tips" is due solely to increased enzyme activity or entirely or in part to accumulation of substrate during the period of regeneration.

Raads & Söding (139, 140) found that dehydroascorbic acid, methylene blue, and, in some cases, even hydrogen peroxide stimulated the growth of *Avena* coleoptiles considerably. They suggest that these agents stimulate the formation of auxin from a precursor. If at all acting on the auxin-system, these substances might stimulate one of the oxidative steps in the conversion of tryptophan to IAA. [Wetmore & Morel (177) let auxin diffuse from *Equisetum* tissue to agar blocks and found that the presence of reduced (?) ascorbic acid in the receiving blocks greatly increased the curvatures ob-

tained in the *Avena* test, presumably by counteracting a polyphenol oxidase.]

Identification of indoleacetaldehyde.—Indoleacetaldehyde was suggested as an intermediate in the formation of IAA from tryptophan by fungi as early as 1937 (175 p. 115), but no experimental evidence for its occurrence in fungi or higher plants was available at that time. Evidence for its occurrence in extracts of higher plants was presented by Larsen (96), Hemberg (72), and Gordon & Nieva (55). In view of these results, the early reports on the presence in plant extracts of a neutral growth substance (93, 102) were evidently dealing with indoleacetaldehyde. Neutral, growth-promoting substance present in plant extracts (21, 39, 172) is now generally believed to be indoleacetaldehyde.

The evidence for the identity of the neutral substance with indoleacetaldehyde is of various kinds. The substance is neutral, but can be converted to an acid by soil (55, 56, 96, 172), Schardinger enzyme (55, 72, 96), and leaf extracts (56). This acid has been identified as IAA by its activity in the *Avena* test and by determination of its diffusion velocity in agar and its sensitivity to hot acid and alkali (96). The diffusion velocity of the neutral substance in agar agrees with what would be expected for a nondissociated substance having the molecular weight of 159 (96). The aldehyde nature of the neutral substance was deduced from the facts that the conversion to acid by soil requires oxygen (96) and that the Schardinger enzyme is an aldehyde dehydrogenase. The most conclusive evidence for its aldehyde nature was presented by Gordon & Nieva (55) who showed that it could be irreversibly inactivated with dimedon (which does not react with ketones) and that it formed an addition product with sodium bisulfite from which it could be regenerated with a 45 to 90 per cent recovery by the addition of soda. This reaction takes place only with aldehydes and with aliphatic methyl ketones and ketones containing two methylene groups adjacent to the carbonyl group.

However convincing this evidence is, it is still indirect, and the actual isolation and chemical identification of indoleacetaldehyde from plant material is still lacking. Such a task is hampered by the fact that this aldehyde has not yet been synthesized. Personal communications from various chemists who have attempted the synthesis indicate that indoleacetaldehyde is very labile. Particularly, it seems to polymerize very rapidly as its concentration increases. Quantities sufficient for physiological experiments, however, may be easily prepared from tryptophan and isatine (96). The neutral, *Avena*-active reaction product thereby obtained has been identified as indoleacetaldehyde by the procedures outlined in the preceding paragraph (but not including the dimedon and bisulfite reactions). The yield is of the order of 1 to 2 per cent only.

Conversion of indoleacetaldehyde to indoleacetic acid.—It was originally believed that indoleacetaldehyde functioned as such as an auxin, because it produced curvatures directly in the *Avena* test (39, 72, p. 204, 96). Auxins are defined on the basis of this property. It was found later, however, that indoleacetaldehyde (prepared from tryptophan) is very rapidly con-

verted to IAA in excised coleoptiles and in coleoptile juice (98). The quantitative data obtained are entirely consistent with the view that indoleacetaldehyde is a precursor of IAA and produces curvatures in the *Avena* test only after enzymatic conversion to IAA. Furthermore, Gordon & Nieva (55, 56) demonstrated the presence of an enzyme system, capable of converting indoleacetaldehyde to IAA in breis and protein preparations obtained from pineapple leaf bases (the indoleacetaldehyde was a natural substance purified via the bisulfite addition product). Wildman *et al.* (184) failed to obtain any conversion of indoleacetaldehyde to IAA by enzyme preparations from spinach leaves, although these preparations produced IAA readily from tryptophan. The synthetic preparation of indoleacetaldehyde used, however, may have contained very little, if any, of the desired substance. Juice from *Artemisia* roots is capable of converting naphthalene acetaldehyde to naphthaleneacetic acid (1).

Amounts of indoleacetaldehyde have been expressed in terms of amounts of IAA obtained after treatment with soil (55, 56, 96, 97, 172). Owing to losses occurring during the treatment, this procedure is unsatisfactory. An enzyme system, capable of converting indoleacetaldehyde and naphthalene acetaldehyde to their respective acids is present in coleoptile juice (98, 99). [Naphthalene acetaldehyde has been shown previously to be active in the *Avena* test (97) and the pea test (168, p. 298). Its synthesis is described by Jensen and his co-workers (77, 78).] Two moles of naphthalene acetaldehyde yielded 1 mole of naphthaleneacetic acid when treated with coleoptile juice (99). The auxin activity of an arbitrary amount of indoleacetaldehyde was increased five-fold by enzymatic conversion of the aldehyde to IAA (98). Assuming identical behavior of the two aldehydes, it was concluded that in order to produce a given curvature (5°) in the *Avena* test, 9 to 10 times as much indoleacetaldehyde as IAA is required. This value may be used for expressing quantities of unconverted indoleacetaldehyde in absolute units. If growth inhibitors are present in a plant extract it may be preferable to convert indoleacetaldehyde to IAA before bio-assay. Coleoptile juice or enzyme preparations made from the juice would be suitable for such conversions. The above-mentioned experiments (99) seem to indicate that the conversion process in coleoptile juice is a dismutation. The conversion in intact coleoptiles may be either an ordinary dehydrogenation or a dismutation.

Formation of indoleacetaldehyde from precursors.—It was shown by Gordon & Nieva (56) that tryptophan can function as a precursor for indoleacetaldehyde. A considerable proportion of the growth substance formed from tryptophan in disks, breis, and enzyme preparations made from pineapple leaves was indoleacetaldehyde. Unpublished experiments by the reviewer showed a similar formation of both indoleacetaldehyde and IAA when tryptophan was added to the juice of etiolated pea epicotyls. Both, however, finally disappeared under the influence of the IAA-oxidizing enzyme in the juice (155).

As possible intermediates between tryptophan and IAA, tryptamine or

indolepyruvic acid (56, 96, 149, 184) have been suggested. Tryptamine was found in *Acacia* by White (179). Tryptamine can be converted to indoleacetaldehyde (and IAA) by disks and enzyme preparations from pineapple leaves (56). Similar materials from spinach failed to produce IAA from tryptamine (184). Interesting examples of the formation of amines from ring-containing amino acids were reported by Werle & Raub (176) who demonstrated the capacity of living leaves of spinach to form histamine from histidine and of *Sarothamnus* to form oxytyramine from dioxypyphenylalanine. Breis and extracts failed to carry out the decarboxylation.

Indolepyruvic acid decomposes spontaneously in aqueous solution to form both indoleacetaldehyde and IAA (56, 184). The reported auxin activity of indolepyruvic acid, 1 to 6 per cent that of IAA (85, 184), is probably due to such decomposition. Existing data, after correction for the spontaneous changes, show that living leaf disks are able to increase the rate of formation of IAA (56, 184) and indoleacetaldehyde (56). Enzyme preparations from spinach (184) reduced the IAA level in a solution of indolepyruvic acid, while similar preparations from pineapple (56) increased the level of IAA and lowered the level of indoleacetaldehyde.

The intermediary formation of indoleimino acid (see scheme p. 179) is entirely hypothetical, but would be in accord with general views on oxidative deamination in animal tissues.

In view of the results reported the question of the intermediates between tryptophan and indoleacetaldehyde must be left open, but the hypothesis that IAA in higher plants arises from tryptophan through indoleacetaldehyde has considerable evidence to support it.

An objection against this hypothesis, however, might be raised on the basis of the extremely low yields (in general much less than 1 per cent) of growth substance formed from a given amount of tryptophan, tryptamine, or indolepyruvic acid. An exception is the formation of IAA from indolepyruvic acid by spinach leaves (184), which at 4 hr. amounted to 9.4 per cent. The spontaneous formation of IAA at the same time (in a not quite comparable experiment) was 2.1 per cent. The fact that such low yields are obtained might indicate that the starting materials are utilized by the plant, mainly in other reactions such as the formation of niacin (45, 69, 81, 153, 158, 188) or that a considerable part of the growth substances formed is again inactivated. The yield of IAA by conversion of indoleacetaldehyde is much higher as judged from the conversion experiments of Gordon & Nieva (56) and from model experiments with naphthalene acetaldehyde (99).

IAA or an IAA-like substance is formed from tryptophan in man and excreted in the urine. The formation (which may be carried out by intestinal microorganisms) seems to be influenced by certain vitamins (pyridoxine, thiamine, riboflavine, folic acid) which decrease the excretion (145). In *Neurospora*, pyridoxine or a derivative thereof has been shown to participate, probably as a coenzyme, in the synthesis of tryptophan (167). Pyridoxine has not so far been shown to participate in the decarboxylation of tryptophan but seems to stimulate the conversion of tryptophan to niacin

in certain animals and also to influence tryptophan metabolism in other ways (147a, 157).

Mineral nutrition and formation of growth substances.—The studies by Skoog (150) on the relationship between zinc nutrition and auxin formation in the tomato plant have been extended by Tsui (166) who, for several weeks, followed the content in leaf and stem of four forms (or types) of auxin: (a) free auxin (extractable with ether in 24 hr. at 15°C.); (b) auxin released by alkaline hydrolysis; (c) by acid hydrolysis; and (d) by incubation with trypsin at a pH value of 8. The content (on a weight basis) of all four forms increased with age in normal plants and decreased in the zinc deficient ones. Addition of zinc to the culture solution caused a significant rise of free and trypsin-released auxin which was evident two days after the addition. After two more days the level of auxin released by alkaline and acid hydrolysis went up. In zinc-deficient plants, the level of free auxin, unlike that of the three other forms, showed an initial increase with age before the decrease mentioned above. The content of tryptophan followed a similar course, but its maximum preceded that of free auxin. Addition of zinc increased the level of tryptophan and also of tyrosine. The author (166) concludes that zinc is required directly for the synthesis of tryptophan. Zinc probably has still other functions. The renewed synthesis of tryptophan upon addition of zinc may be just one symptom of the general recovery of the plant. Nason *et al.* (121, 122) found that the activities of alcohol dehydrogenase and of the tryptophan synthesizing enzyme were almost absent in zinc-deficient mycelium of *Neurospora*, while diphosphopyridine nucleotidase activity was increased 15-fold as compared with mycelium grown on a complete medium. The results of Tsui support the view that there is a close genetic relationship between tryptophan, free auxin, and auxin which can be released by tryptic digestion. The forms of auxin which are released by acid and alkaline hydrolysis appear to be formed later than the other two. Zinc-deficient plants, being very low in auxin, but having the capacity to resume the synthesis upon the addition of zinc, evidently constitute an excellent material for the study of the formation of the various types of auxin.

Skoog (150) succeeded in partially relieving the early symptoms of zinc deficiency in tomato plants by the addition of low concentrations of IAA. Eaton (38) reported a corresponding effect of IAA on boron-deficient cotton plants. MacVicar & Tottingham (110), however, were unable to confirm Eaton's results, either with cotton, sunflower, soybean, or tobacco. Gustafson (60) confirmed the general view that well-fertilized plants have higher auxin contents than plants grown in poor soil and also reported other observations on the influence of external and internal factors on the auxin level.

PRODUCTION AND OCCURRENCE OF GROWTH SUBSTANCES DURING CERTAIN PHASES OF DEVELOPMENT

One of the goals of auxin research is to elucidate the role of the various types of auxin and inhibitors in physiological processes. Unfortunately, our methods for separation and determination of these regulators still need im-

provement, and some of the physiologically active substances in extracts may even be artifacts. Owing to such difficulties, several of the interpretations presented in this section in an attempt to correlate growth with occurrence of regulators will probably need revision in the future.

Grass seedlings.—The discussions of the auxin-relationships in kernels and seedlings of grasses strikingly reflect the problems and controversies in the whole auxin field. On the basis of older and recent reports, a picture like the following may tentatively be drawn. During the development of the grain the endosperm—mainly the aleurone cells (70)—accumulate auxin. With ripening, the total content of auxin decreases (5, 70, 138). In the mature grain most of the remaining auxin is present in a bound form. During the earliest stages of germination the bound auxin is set free (67), but the liberated auxin, probably IAA, disappears within a few days (7, 67). It seems to become inactivated, either in the scutellum (43) or in the first internode (67), and move upward in the inactive form. The transport form has been assumed to be the inhibitor-auxin complex (43) or indoleacetaldehyde (63). Having reached the coleoptile tip, the inactive form becomes reactivated and moves downward. One hypothesis assumes that IAA—free or bound (respectively active or inactive)—is the only auxin involved in these transformations. Another, advocated by von Guttenberg (63, 67), assumes two parallel systems of transformation, one involving IAA, the other auxin *a*. According to the latter hypothesis, the final liberation of auxin *a* in the coleoptile tip is caused by IAA which has first itself become “activated” in the tip. This view of the function of IAA, however, is far from being generally accepted. The characteristic distribution (181) of the enzyme system which partially converts tryptophan to IAA is difficult to fit into either of these hypotheses. If, however, the inactive transport form of auxin contained a derivative of IAA, this might be the natural substrate for the enzyme. The meaning of the observations on which the two hypotheses are based can hardly be properly apprehended until more definite information about the chemical nature of the participating types of auxin is at hand. The problem, however, seems open for further experimentation, even without the availability of pure preparations of auxin *a*.

The question of the amount of auxin (computed as IAA) present in or extractable from *Avena* coleoptiles needs a few comments. Moewus (117), using the cress-root test, found 0.1 $\mu\text{g.}$ of IAA per coleoptile tip (10 mm.). This quantity is probably made up largely of bound auxin, since the juice in which the auxin was determined was kept for 48 hr. (at 4°C.) before the test, which itself lasts 17 hr. (at 27°C.). Moewus' claim that the content of 0.1 $\mu\text{g.}$ of IAA per tip is in accord with Thimann's (159) values rests, however, on the erroneous assumption that one *Avena* unit (AE) equals 25×10^{-9} gm. of IAA. The fact is that 1 gm. of IAA equals 10 to 25×10^9 AE, 1 AE being 4 to 10×10^{-11} gm. of IAA. Thimann found 4.5 plant units, or 1.8 to 3 AE of auxin per 10-mm. tip. Using these values we find 0.7 to 3×10^{-4} $\mu\text{g.}$ per tip. From fresh coleoptile juice about 1.7×10^{-4} $\mu\text{g.}$ of IAA per 25-mm. tip could be shaken out with ether (98). From Wildman & Bonner's data

(181) it may be computed that about 1.6×10^{-4} $\mu\text{g.}$ of IAA per 15-mm. tip is extractable with cold ether after lyophilization. If the ether-extractable auxin comprises 20 per cent of the total diffusible auxin (181) the latter amounts to about 10×10^{-4} $\mu\text{g.}$ per coleoptile tip. Moewus' value, therefore, seems to be about 100 times as high as those obtained elsewhere [cf. (140a)].

Potato tubers.—In sprouting potato tubers, as in germinating seeds, we are dealing with stored auxin (or auxin precursors) and growing organs requiring auxin. Also here, the concept of a bound storage-form of auxin which yields IAA via indoleacetaldehyde is supported by several observations. Moewus (115) found that 99 per cent of the auxin in nonsprouting potato tubers was present in a bound form. This bound form and the inhibitor-auxin complex (43, 111) may be identical. Hemberg (72) showed that the periderm layer of sprouting tubers yields more indoleacetaldehyde than that of nonsprouting ones. [Exposing potato peelings to air increased their content of indoleacetaldehyde and IAA. Dostal (33) found that wounding potato tubers by burning increased their auxin content.] The level of IAA in the periderm layer reaches a maximum just before sprouting. Potato tubers also contain a number of growth-inhibiting substances (20, 72, 111). According to Hemberg (72, 73), the cessation of dormancy shows a closer correlation to variations in the content of such inhibitors than to changes in the auxin level. On the other hand, the elongation of the shoots after sprouting has started must require auxin. This auxin is probably mobilized from a bound storage-form. The interrelationships between IAA and the acid-stable auxin (auxin *a*?) reported both by Hemberg (72) and the German group (43, 111) is not clear. Meyer (111) reports a lower content of total auxin with a higher ratio of free to bound auxin in leaf-roll infected tubers as compared with healthy ones. Tubers infected with *Phytophthora* contained considerably more auxin than uninfected tubers (33).

Buds, leaves and stems.—Brandes & van Overbeek (21) found that the breaking of dormancy in buds of sugarcane was associated with a lowering of the level of free auxin in the nodes. The authors hesitate, however, to postulate a causal relationship between the two phenomena. Hemberg's results with potato (72, 73) and *Fraxinus* (74) indicate that specific growth-inhibiting substances may play a more important role than auxin in dormancy of buds. The inhibitor-auxin complex, mentioned previously, might be worth considering from the point of view of dormancy.

Various observations on the growth of young buds seem to point to what may turn out to be a general pattern in the auxin relationships of growing buds. It appears that the leaves do not deliver auxin, but probably furnish a precursor, which is converted to auxin by the young, growing stem. This view is supported by the findings of Gunckel & Thimann (58) who failed to obtain diffusible auxin from leaves or apical meristems of young, elongating "long shoots" of *Ginkgo*, but did obtain auxin from the rapidly growing internodes. Also Hatcher's (71) results with apple and plum are in accord with the above suggestion. Van Overbeek *et al.* (133) actually found that the

bases of young leaves (of pineapple) contained little free auxin but large amounts of bound (precursor) auxin, while the situation was the reverse in the stem. In the fern *Adiantum*, however, the young leaves did yield diffusible auxin (178), the highest yields being obtained from the tips.

Von Witsch (186) and Leopold (101), working with *Kalanchoe* and *Coleus*, respectively, found the content or production of auxin in the leaves to be increased by longer photoperiods. Von Guttenberg & Kröpelin (66) showed that the yield of auxin from *Phaseolus* leaves is subject to diurnal variations (minimum at midnight). The relationship between flowering, endogenous leaf-movements and auxin production is being studied by Laibach & Kribben (90, 91). Upon darkening of one cotyledon of *Cucumis*, and either removing or leaving the opposite one, curvatures appear in the hypocotyl. The magnitude of these curvatures, which show rhythmic fluctuations, is taken as a measure of the rate of export of auxin from the cotyledons (the rate is higher in darkness than in light). In leaf-movements a rise of the leaf is assumed to be associated with a high, a lowering with a low rate of auxin export. Records of the leaf-movements in *Arabidopsis*, *Urtica*, and *Coleus* are presented and taken as indications of parallel changes in auxin export.

Betzler & Bünning (12) removed one leaf from a young symmetrical pair and placed the plant in darkness. The stem portion below the remaining leaf showed diurnal variations in curvature which were taken as indications of parallel variations in export of auxin from the leaf. On this criterion the soybean (var. "Peking"; short-day plant) showed a minimum of auxin yield at midnight and maximum at 4 p.m., while *Hyoscyamus* (long-day plant) showed a minimum at 2 p.m. and a maximum at 6 a.m. The relationship between flowering, leaf-movements, and photo-period is striking, as pointed out by Bünning (23, 24, p. 385). The connection of these phenomena with parallel variations in the auxin level is highly probable and not incompatible with our scanty knowledge of hypo- and epinasty. Before these views can be generally accepted, however, further analyses, particularly additional quantitative auxin determinations, seem to be required.

Linser (103) extracted various leaves with alcohol and reports on the content of growth-promoting and growth-retarding substances in the extracts. The variations in phytohormone content in stem apices of celery were followed by Clark & Wittwer (28). Künning (89) compared the auxin activity in extracts of cambium, fruits, and leaves of *Tilia* with the capacity of these extracts and of synthetic compounds to induce cambial activity in *Phaseolus* and *Helianthus*. He concludes that cambial activity under natural conditions is regulated by the cooperation of auxins and specific substances which induce cell divisions.

The auxin-relationships in terminal hooks of etiolated sprouts of potato, pea, and broad bean were studied by Dostal (36) who found about twice as much diffusible auxin in the lower as in the upper side of the hooked portions of the stem. The auxin concentration in the lower side is evidently

supra-optimal for elongation. Tips of mechanically bent rhizomes of *Circaea* (35) showed a higher tissue tension and a higher auxin content in the concave than in the convex half.

Roots.—Warmke & Warmke (172) followed the changes in free (acid), neutral, and bound auxin for 8 days during the regeneration of sections of *Cichorium* roots. The level of neutral auxin was more than three times as high as that of either of the two other forms. The level of all three forms decreased immediately after cutting the sections. Neutral auxin started to increase after 6 hr., bound auxin after 12 hr., while free auxin remained low for about 48 hr. In the case of bound auxins these changes occurred to the same degree in both ends of each section. Neutral and free auxin, on the other hand, became temporarily more abundant in the distal (root-forming) than in the proximal (shoot-developing) end of the section. Kulescha & Camus (88) probably determined the sum of all three types of auxin in regenerating chicory-root sections. They, too, found a decrease soon after cutting. This decrease was more pronounced in the proximal than in the distal end, thus leading to a transitory excess of auxin in the latter. The results of both groups of workers indicate that shoot-differentiation is associated with a low, root-formation with a high content of auxin. In addition to auxins, growth-inhibitors seem to be operating. These inhibitors may either reduce the apparent auxin level at the proximal end (172) or inhibit buds formed at the distal end (88). Studies on the correlative inhibition of the plumule and promotion of the radicle in germinating peas were made by Dostal (32) who concludes that the correlation is mediated by auxin liberated from the cotyledons.

It is generally stated in auxin literature that the natural concentration of auxin in roots is supra-optimal since addition of auxin leads to retardation of growth. A recent confirmation of this current view was contributed by Naundorf & Vallmitjana (125) with the interesting new finding that washing of the cotyledons of *Vicia Faba* (reducing the supply of auxin to the root) led to increased growth of the main root. Corresponding results have been obtained with grass seedlings (124).

Naundorf (123) showed that illumination of the roots of *Helianthus* increased their auxin content and growth rate. This finding, together with the growth-promoting effect of direct application of auxin to intact roots reported recently by Moewus (113, 114, 117, 118), Linser (104), and Ashby (1), and previously by others (109, 123, 163), indicate, against current views, that at least some normal, intact roots have a sub-optimal auxin concentration. In geotropism, if such roots build up an inhibitory auxin concentration on their lower side, we must assume that the concentration passes through a stimulatory range before reaching inhibitory values.

Burström (25a, 26) recognizes two different phases of growth in wheat roots [cf. (151)]. The first phase is characterized by increasing, the second by decreasing elasticity of the cell wall. All concentrations of auxin stimulate the first phase, while higher concentrations inhibit the second one. Over-all acceleration by low concentrations is ascribed to stimulation of the first phase and lack of inhibition of the second phase. According to these results,

the natural level of auxin in wheat roots is sub-optimal for the first, but supra-optimal for the second phase of growth. Lundegårdh (108a) contends that the concentration of auxin in the surface layer of wheat roots corresponds to 10^{-9} M of IAA.

Tubers.—Deusse (31) found no (?) auxin in the stems of certain tuber-forming species of *Brassica* until after tuberization had begun. He supports the conclusion of Podesva (137) that although the growth of the tubers is regulated by auxin (from the leaves) the initiation of tuberization depends on specific tuber-inducing substances.

Enlarging fruits.—The hypothesis that the auxins responsible for the enlargement of fruits comes from the maternal tissue or developing seeds (130), rather than from the pollen, has been supported by various experimental results. Wittwer (187) and Hatcher (70) found no detectable auxin in the developing ovules of cereals but large amounts shortly after fertilization. In maize the auxin content of the young seeds reached a peak within 10 to 15 days and thereafter decreased again. The auxin production in developing grains is probably responsible for the "spontaneous" parthenocarp in adjacent, unfertilized pistils (22). Ripe pollen of rye and maize seems not to be a particularly rich source of auxin and neither is pollen from diploid or triploid apples (100). (The auxin activity of the apple pollen showed no correlation with germinability or pollinating value.) A direct influence of pollen on the production of auxin in ovary tissue was demonstrated in tobacco by Muir (120) who showed that addition of an aqueous extract of ground pollen to dried tissue of ovaries resulted in the release of considerable quantities of auxin from the ovary tissue. Pollen and the ovary tissue contained little or no auxin. The ovaries, however, contained bound auxin, which could be released enzymatically or by alkaline or acid hydrolysis. Pollen contained small amounts of bound auxin.

Luckwill (106, 107, 108) showed that water-extractable auxin in young apple seeds is produced, not by the embryo, but by the endosperm. No auxin, however is detectable in the young seeds during the 3 to 4-week period in which the endosperm remains in the free-nuclear stage, although the fruits grow rapidly during this period. The auxin required for this growth must come from tissues outside the seeds. As cell walls are formed in the endosperm auxin appears. The auxin content of the developing seed, however, decreases again as the rapidly growing embryo draws on the endosperm tissue. After the development of the embryo, the auxin content rises considerably but only to drop to a low level again as the fruit enlarges. The minimum values of auxin content in the seeds seem to be closely related to periods of fruit drop. Furthermore, dropped fruitlets contained fewer seeds and less auxin per seed than fruitlets which stayed on the tree.

Moewus (119) found that ripe and unripe cherries contained about the same amount of auxin per ml. of juice. In the unripe ones, however, about 90 per cent of this auxin was in the bound form.

Nitsch (126, 127) followed the production of free auxin in developing achenes of strawberry. A small amount of auxin was found three days after

fertilization. Nine days later 35 times as much was present, but the content dropped subsequently to about one third of the peak value. The receptacle of the strawberry showed no auxin content at any stage of development, but its enlargement showed close correlation to the number of fertilized achenes and to their production of auxin.

Tissue cultures.—Certain plant tissues are able to grow in synthetic media without an exogenous supply of auxin. This fact indicates the capacity of such tissues to synthesize auxins from other materials. These phenomena are discussed by White (179a).

INACTIVATION OF GROWTH SUBSTANCE

Oxidation of indoleacetic acid.—The literature on enzymatic breakdown of growth substances was briefly summarized by Tang & Bonner (155) who made a detailed study of the IAA-inactivating enzyme system present in etiolated pea epicotyls. This work was reviewed by Skoog (151). A further study of the same enzyme system was made by Wagenknecht & Burris (171) who confirmed the following observations of Tang & Bonner: (a) oxygen is necessary for the inactivation of IAA as shown previously for bean epicotyls (95); (b) one mole of carbon dioxide is evolved per mole of oxygen consumed; (c) the pH optimum for the reaction lies between 6 and 7; and (d) the reaction is inhibited by cyanide.

Wagenknecht & Burris (171), however, found no inhibition of the reaction by the addition of carbon monoxide, neither in light nor darkness. They present evidence that the inhibition reported by Tang & Bonner (155) was caused by oxygen deficiency in the mixture of carbon monoxide and oxygen. That lowering of the oxygen tension was less inhibitory in light than in darkness (155) may be explained by the inactivation of an enzyme-inhibitor by light [cf. (47)]. On the basis of their results with specific inhibitors, particularly sodium diethyldithiocarbamate, Wagenknecht & Burris conclude that the IAA-oxidizing enzyme in pea epicotyls is a copper-protein rather than an iron-protein as was assumed by Tang & Bonner. Another point of divergence is the specificity of the enzyme. Tang & Bonner (155) concluded from colorimetric determinations (Hopkins-Cole reaction) that indolepropionic and indolebutyric acids were not inactivated by the enzyme, while Wagenknecht & Burris, using manometric techniques, found that the two compounds were oxidized by the enzyme. With indolepropionic acid, however, only 0.2 moles of carbon dioxide were released per mole of oxygen consumed, indicating a mechanism of breakdown different from that involved in the oxidation of IAA. The reported specificity of the IAA-oxidizing enzyme in pea epicotyls has been used as a support for the view that certain auxin preparations, sensitive to the enzyme, contain IAA (55, 119).

In the root sap of the yellow wax bean Wagenknecht & Burris (171) found an IAA-oxidizing enzyme with properties very similar to those of the enzyme found in etiolated pea epicotyls. Enzymes from both sources were stimulated by manganese, although the amounts of manganese required

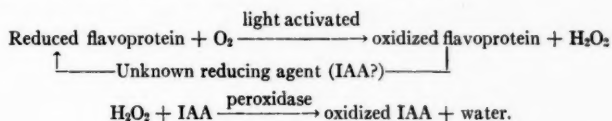
for maximal stimulation differed. The optimal substrate concentrations were also different.

Excised (98) and lyophilized (156) *Avena* coleoptiles inactivate IAA, but the oxidizing enzyme is not present in coleoptile juice, which, on the other hand, contains the indoleacetaldehyde-converting enzyme (98).

Mitchell, Burris & Riker (112) studied the influence of various growth substances on the respiration of several plant tissues. In all cases the effect was an inhibition of respiration except that the respiration of bean root tissue was stimulated by IAA (but not by naphthaleneacetic acid and 2,4-D). However, in the presence of diethyldithiocarbamate (which inhibits the IAA-oxidizing enzyme without affecting normal respiration) the usual inhibition of respiration was found. These results imply that some caution should be exerted in the interpretation of reports of stimulation of respiration by IAA. The IAA may merely have served as a substrate for the oxidizing enzyme.

The end product formed by the oxidation of IAA is a neutral, ether-soluble substance which still contains an intact indole nucleus (155, 171) and shows a carbonyl character (171). These properties, and the fact that the R.Q. of the reaction is equal to 1, make it likely that the end product is indolealdehyde.

Galston and his co-workers (44, 47 to 50) suggest a connection between the action of the IAA-oxidizing enzyme and the photoinactivation of IAA *in vivo* and in impure plant extracts. Their experimental results may be discussed with reference to the following scheme (47):



Galston and his co-workers found that riboflavin sensitized the non-enzymatic photooxidation of IAA *in vitro* (44, 48, 50). This work was reviewed by Parker & Borthwick (134). It was also found (47) that light stimulates the IAA-oxidizing enzyme system in etiolated pea epicotyls and that the action spectrum for this stimulation was very similar to that of the riboflavin-sensitized photooxidation. Since the photoreceptor, giving the flavin spectrum, is heat-labile and non-dialyzable, it is considered to be a flavoprotein. Light seems to remove a naturally occurring substance which partially inhibits the flavoprotein enzyme (which thereby becomes "activated"); but light is not indispensable for the over-all oxidation of IAA (155, 171). The activity of the enzyme was inhibited by catalase and by various metals (such as manganese) which react with hydrogen peroxide. This led to the conclusion that the flavoprotein enzyme produces hydrogen peroxide, which in turn oxidizes IAA. In the presence of catalase or manganese, blue light accelerates the reaction, presumably by removing the

natural inhibitor of the hydrogen peroxide production. The substrate for the flavoprotein enzyme is not known. It is suggested that it may be IAA which would thus be yielding the oxidant for the inactivation of more molecules of its own kind through the next step, the action of a peroxidase.

The mediation of a peroxidase in the inactivation was concluded from experiments which showed that the whole enzyme system is stimulated by the addition of hydrogen peroxide in the dark (where the production of this compound is limited by the natural inhibitor) and by crystalline horse-radish peroxidase in the light (49). The whole enzyme system could be fractionated into a flavin fraction and a peroxidase, the latter active toward IAA and conventional peroxidase substrates. A point which cannot be judged from the brief report (49) is whether the spontaneous oxidation of IAA by hydrogen peroxide was considered in the experiments with this compound. All of the known peroxidases are iron proteins. The conception of the IAA-oxidase as a peroxidase, therefore, is in disagreement with the view of Wagenknecht & Burris (171) that this enzyme is a copper-protein. A further difficulty arises from the controversial reports on the effect of manganese on the enzyme system (47, 171).

The product formed from IAA by the action of free riboflavin, light, and oxygen is thought to be a condensation product of several cleaved indole rings (47). If the final product formed by the IAA-oxidizing enzyme system is indolealdehyde, as indicated above, and if a peroxidase participates in the reaction, then probably also a carboxylase is involved.

It was shown by Goldacre (52) that 2,4-D increases the rate of destruction of IAA by the enzyme system in etiolated pea epicotyls. This observation was confirmed by Galston *et al.* (49), who showed that 2,4-D reverses the catalase inhibition but not the cyanide inhibition of the whole system. They conclude, therefore, that 2,4-D influences the flavoprotein enzyme.

A heat-stable factor inhibiting the activity of the IAA-oxidizing enzyme system was found in all species and tissues tested (52, 156). Green pea seedlings contained considerably more of the inhibitor than did etiolated tissue, buds were a richer source than stems, and illumination promotes the formation of the inhibitor. If Galston's suggestion (47, 49) that this inhibitor is identical with the inhibitor of the flavoprotein enzyme is correct, we are here dealing with a substance the formation of which is promoted by light but whose activity is reduced by illumination. The action spectrum for the formation of the inhibitor, however, is unknown and may be different from that of its "removal" by light.

The physiological role of the IAA-oxidizing enzyme was sought by Tang & Bonner (156) and by Goldacre (52) in the maintenance of a constant level of IAA in the tissues (particularly in roots) which would otherwise become flooded with IAA owing to its steady production from precursors. The enzyme was present in most etiolated tissues and in roots (except carrot roots), but first (156) reported to be absent from green tissues. Bonner (15, p. 451), however, mentions that green leaves do contain the enzyme.

Galston (46, 47, 48) suggests that the flavoprotein-catalyzed photo-

inactivation of IAA may be responsible for the group of phototropic curvatures which is caused by unilateral inactivation of auxin (as distinct from curvatures caused by redistribution of auxin). The general belief so far has been that carotenoids were the photoreceptors in those reactions. The literature on phototropism was reviewed recently by Galston (46), Bünning (24a), and Schrank (147).

The question of the prevention of certain phases of etiolation, especially of suppression of hyper-elongation of internodes in etiolated plants, appears to be closely related to photoinactivation of auxin. The flavoprotein-enzyme in etiolated peas, however, cannot be solely responsible for the prevention of hyper-elongation of the epicotyls since red light has been shown to be much more effective than blue in this respect (135, 174). Bünning (24, pp. 366-67) points out that the suppression of etiolation symptoms can be accomplished in older plants with blue-violet radiation and that red light is more effective only in young seedlings. Bünning contends that chlorophyll (or a related compound), which may first be formed photochemically, sensitizes auxin destruction in such seedlings under the influence of red light. A further discussion of the etiolation phenomena and of phototropism would fall outside the scope of this review.

The inactivation of IAA by x-rays and γ -rays was studied by Gordon & Weber (57). In the presence of oxygen the breakdown seems to be due chiefly to ring-opening and apparently is an oxidation. Under very low oxygen tension inactivation is slow and the point of attack seems to be in the side chain. The studies of King (84) indicate the formation of a growth-inhibitor by x-ray treatment of tobacco callus.

Reversible inactivation of indoleacetic acid.—Moewus (115) added IAA to juice prepared from potato tubers. After 24 hr. of incubation, he found that the juice did not show higher auxin activity than untreated juice. The IAA, however, was liberated by treatment with pancreatin. Evidently the IAA had not been destroyed, but only bound (presumably to proteins) and thereby inactivated. This fixation of IAA did not take place in boiled potato juice. Juice from unripe cherries (119) showed a similar behavior, whereas juice from ripe cherries was unable to bind IAA. Potato and cherry juice apparently do not contain the IAA-oxidizing enzyme system. A reversible fixation of IAA may also take place in *Avena* coleoptiles (16).

Von Guttenberg & Steinmetz (68) studied the relationship between auxin and the effect of ethylene (illuminating gas and apple emanations) on growth. *In vitro* ethylene had no effect at all on IAA but reduced considerably the auxin activity of diffusates from coleoptile tips and of *Coleus* extracts. The authors conclude that ethylene inactivates auxin *a* (but not IAA) by combining loosely with the molecule of auxin *a*. (The original auxin activity of the diffusate or extract can be almost completely restored by removing the ethylene by boiling or evacuation.) This interpretation is not necessarily invalidated by the findings of Dalbro & Müller (29) that ethylene, in all concentrations tested, retarded the growth of pea roots. If the natural auxin concentration in the root is sub-optimal (see previous section) retardation

of growth is to be expected if part of the auxin is inactivated. Dostal (34) reported an increase of about 50 per cent in the auxin content of potato tubers following treatment with illuminating gas. The increase is probably an indirect effect caused by ethylene and other gases.

Methods for the determination of ethylene have been reviewed by Biale (13) and a biological method has been described by Dalbro & Müller (29).

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HERBICIDES AND SELECTIVE PHYTOTOXICITY

PART I

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INTRODUCTION

The field of herbicides and phytotoxicity continues to command the widespread interest of workers in all parts of the world. In spite of the fact that this subject was fully reviewed by Norman *et al.* (209) in the previous volume, the present review contains more than 300 new references. With such a volume of work ranging over the many subjects relating to the study of phytotoxicity it is almost inevitable when the joint authors work in different laboratories that there be some dichotomy in the preparation. In consequence, the first author has been most concerned with the dinitro-alkyl-phenols and general aspects of phytotoxicity (Part I) and the other two authors with plant growth regulators (Part II).

In spite of the striking developments in the production of new herbicides in the last decade and their extensive application in agricultural practice, the general approach is still empirical, involving a high degree of speculation in the selection or synthesis of possible new compounds, followed by trial and error tests in the laboratory, greenhouse, and field. At the present time, progress is still rapid, but there is no doubt that it will be further accelerated when the principles of toxic action are better understood. Mitchell (196) has discussed the importance of physiological studies, while it has been emphasized in a recent review [Blackman (28)] that the analysis of selective toxicity demands integrated studies of the main factors which may operate. Selective action may not only involve differences in toxicity at the cell level but also differences in the quantities reaching the site of toxic action. These amounts, in turn, will be dependent on differences in root absorption, retention by the shoot, penetration into the plant, transport within the plant, and variations in localised accumulation.

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PART I

THE ASSESSMENT OF PHYTOTOXICITY

In the detection or selection of toxic compounds which may have value as herbicides, many attempts have been made to devise routine tests of toxicity. Because the effects of different compounds can be so diverse, it is impossible to assess the toxic properties by a single test in the laboratory or greenhouse. Norman *et al.* (209) listed various techniques and discussed some of their limitations. Most of these screening tests have been devised to evaluate growth-regulating effects similar to those brought about by 2,4-dichlorophenoxyacetic acid (2,4-D or DCPA) or O-isopropyl-N-phenylcarbamate (IPPC or IPC) and, in general, comparisons have been restricted to a single dose level.

The Chemical Biological Coordination Center of the National Research Council (10) report results of this type based on the following tests: (a) inhibition of primary root development of cucumber (*Cucumis sativus*) in which the seed is placed either on filter paper or in treated soil; (b) inhibition of the vegetative growth of the shoot of kidney bean (*Phaseolus vulgaris*) after the compound has been applied in the following ways: (i) the shoot is sprayed with an aqueous or acetone solution, (ii) a band of lanolin paste is placed around the stem at the first internode, (iii) the stem is pierced with an impregnated thread at the second internode; (c) inhibition of the vegetative growth of barley after the plant has either been sprayed with an aqueous solution or the compound applied as a lanolin smear to the upper surface of the first leaf; and (d) inhibition of the vegetative growth of oat seedlings grown in soil to which the compound has been added. In these techniques inhibition is usually measured by comparing the weights of the tissues of treated and control plants after a given time interval. In the barley test plant kill is also considered.

Screening tests of this type, based on different responses such as epinasty, formative effects, and the inhibition of germination have been used by many workers [Norman *et al.* (209)]. Others have measured the effect in terms of either the depression in growth or the percentage mortality after spray applications to plants grown in pots. These last techniques have the advantage that they are equally suitable for estimating the toxicity of compounds which do not come within the category of growth regulators.

Comparisons between compounds based on a single dose level may give misleading results, since for a large number of biological effects the relationship between the effect and varying dosage is sigmoid. Therefore, for accurate comparisons between compounds, varying amounts or concentrations must be employed, so that the level which brings about a given response can be determined and the relative toxicities expressed in terms of the equi-effective dose level. Simon (261) and Blackman (28) in their studies of the relative toxicity of nitrophenols refer to tests in which the toxicity has been expressed as the dose required (a) to produce a 50 per cent mortality of mustard

(*Brassica alba*) seedlings, (b) to halve the frond multiplication rate of *Lemna minor*, (c) to halve the growth rate of the fungus *Trichoderma viride*, and (d) to reduce the respiration rate of yeast by 50 per cent. Audus (13, 14) has described a method for the accurate measurement of root extension growth and has studied the dose response relationships of 2,4-D and coumarin. Wilske & Burström (313), in their comparison of thio-phenoxy acids, grew oats in flowing culture solution and investigated the inhibitory action on root growth and epidermal cell elongation. The test based on the suppression of leaf growth in the kidney bean has been more fully analysed by Brown & Weintraub (37) and they have evolved an improved technique whereby a quantitative evaluation can be made of the "formative" activity of growth regulating compounds. Just prior to the unfolding of the first trifoliate leaf of the bean seedling, application is made to the apical bud and repression of the development of the trifoliate leaf measured in terms of area or weight.

When percentage inhibition in root growth (data of Audus) or the percentage reduction in leaf area (data of Brown & Weintraub) is plotted against the logarithm of the dose level for 2,4-D, then the response is almost directly proportional to dosage over an approximate twenty-fold range. A closer examination of the relationships reveals, however, that they are not linear but sigmoid. Indeed, when the leaf area data given by Brown & Weintraub are transferred to the probit transformation, they fit the linear regression with precision.

Although a monograph on probit analysis is available [Finney (95)] and the methods are extensively used for the precise evaluation of insecticides and fungicides, little attempt has been made to employ them in studies of phytotoxicity since the pioneer study of Cook (58) in 1937. It has been emphasized by Woodford (318), quoting results from the Oxford investigations, that the data for most herbicidal studies, based on the quantal response of death and many qualitative responses, give normal sigmoid dosage response curves that are best treated statistically by probit analysis. Fogg (96), Blackman *et al.* (31), Ivens & Blackman (143), and Roberts & Blackman (239) have used this statistical technique in the assessment of phytotoxicity data.

Such quantitative tests have been primarily evolved for the comparison of the effects of different compounds on the same species, particularly where the aim is to relate differences in toxicity to changes in chemical structure. Where, however, the application is made to whole plants or to isolated tissues, it must be borne in mind that what is measured is an overall effect involving the movement and partition of the substance between the point or points of application and the site of toxic action.

Thus, the complete assessment of a selective herbicide must include a study of each of the intermediate aspects of its selective action, namely, root absorption, shoot retention, penetration, translocation, and accumulation.

For the elucidation of the effects at the cell level, most precision will be obtained when working with simple organisms or small aggregates of cells,

freely exposed to the test solution. In this connection, Ferguson (93) has shown that for systems in equilibrium the thermodynamic activity (partial molar free energy) gives a measure of the toxicity at the site of action within the cell and that it is possible to deduce whether the toxic agent is acting physically or chemically. This valuable approach has not received all the attention that it deserves.

In studies of the relative toxicity of substances applied in aqueous solution another factor of importance is pH. Simon & Blackman (262) have demonstrated that the pH of the test solution affects the activity of weak acids, such as nitrophenols and growth substances. Most valid comparisons are obtained at pHs below the pK values of the compounds, but if the experiments at these pH levels are not feasible an approximate correction for the degree of ionization can be made from the graph given by Simon (260). The effects of pH have been shown to be appreciable in tests with microorganisms, the *Avena* cylinder test, and the pea curvature test, but when a small quantity of the test solution is applied to a large bulk of tissue (aqueous spray applications to whole plants, *Avena* curvature test) the effect of pH is partially or wholly masked.

Solvents used for the dilution of water insoluble herbicides often increase the facility of application, but the interpretation of the results must take into account both direct and indirect effects of the solvent. Redemann *et al.* (236) have shown that unless anhydrous lanolin is specially purified, the contained oxidising agents may destroy the activity of certain growth regulating substances and thereby introduce errors of between ten and a hundredfold in quantitative assays. Again, changes in the composition of the spray solution, such as the addition of surface active or emulsifying agents, may differentially affect spray retention and penetration. Blackman (27) has demonstrated that while with mustard seedlings there is little difference in spray retention between an aqueous suspension and a 10 per cent oil emulsion of 3,5-dinitro-*o*-cresol, in the pea, the retention of the oil emulsion is three times as great.

The proceedings and research reports of the several weed control conferences held in North America during the last year are full of the results of field trials for which details of experimental design or methods of assessment are rarely given. It would, however, appear that where the results of mortality data have been statistically analysed, the analysis of variance has been employed and little attempt has been made to obtain dose-response curves. Experimental techniques for the evaluation of selective herbicides have been discussed by Woodford (317). Statistical methods most suited to the evaluation of quantal responses, such as kill, and the quantitative response of yield, are considered, together with the necessity for the accurate determination of the toxicity of the herbicide, to both the weed species and the crop separately, before an attempt is made to estimate the selective effect on a weed-infested crop. Stahler (270) has essayed to arrive at a single figure for herbicidal efficiency which is based on both the response of weeds and crop plants.

Ennis & Norman (87) point out that systematic errors may well arise in methods of spray application and have put in a plea for more accurately controlled experimentation. Many workers report that for several crops there may be major differences in the liability to herbicidal injury, while there is again evidence that the volume in which the herbicide is applied is another variable (27). Too frequently, details of design, methods of application, the physical properties, and the exact composition of the spray solution are omitted while precise information on the crop, the weeds, and the methods of evaluating results are not given. In consequence, a basis for critical comparison is lacking.

DINITRO-ALKYL-PHENOLS

Although dinitro-alkyl-phenols have been extensively employed as herbicides for a number of years, there is as yet for higher plants scant information on their toxic effects or on the physiological and biochemical factors involved in selective action. However, it is well established that nitro-phenols are toxic to a wide variety of organisms ranging from microorganisms to higher plants and animals; indeed, their toxicity to man demands special precautions in their use in the field [Bidstrup (21), Hunter (141)]. Because of their general toxicity, it seems probable that these nitro-phenols act by interfering with some basic metabolic process and that in consequence information gained from a study with any one organism will help to elucidate the mode of action on another organism [Brian (36)]. It is proposed, therefore, to review the general physiological and biochemical effects of these compounds before considering the latest developments in their use as herbicides.

Biochemical and physiological effects.—In recent years, nitro-phenols have been widely employed in the study of various aspects of metabolism. It has been found that at low concentrations 2,4-dinitro-phenol and 3,5-dinitro-*o*-cresol may double or even treble the respiration and fermentation rates. Peiss & Field (226) cite many examples of such stimulation for microorganisms and animal tissues, while similar observations for higher plants have been made by Bonner (34), Kelly & Avery (154), Newcomb (208), and Stenlid (273).

There is evidence from experiments with microorganisms and animal tissues that the enzymes of normal respiration operate under the conditions of stimulation. The work of Krahle & Clowes (56, 163) and Dodds & Greville (75) has shown that the R.Q. is not changed, while Krahle & Clowes (165) and Bodine & Boell (32) have demonstrated that the stimulated respiration is sensitive to the same inhibitors as normal respiration. For critical discussion of the view held by some workers that nitro-phenols act as accessory reversible oxidation/reduction systems transferring hydrogen from substrates to oxygen, reference should be made to Clowes & Krahle (56) and Pickett & Clifton (231).

At any one pH, concentrations of dinitro-phenol and dinitro-*o*-cresol can be found which will selectively inhibit synthetic processes, while allowing the continuation of respiration. Typical energy-requiring processes so inhibited

are the oxidative assimilation of carbon compounds by microorganisms [Clifton (54)] and the formation of adaptive enzymes by yeast [Spiegelman (269)]. Recently, Stenlid (273) has found that nitro-phenols selectively inhibit the absorption of glucose by wheat. In addition, Clowes & Krah1 (56) have also demonstrated that the cell division of fertilised Echinoderm eggs is inhibited, while Bonner (33) records that the growth of sections of *Avena* coleoptiles is arrested.

The implication of these findings is that in the presence of critical concentrations of nitro-phenols the energy released in respiration and fermentation is not available to promote synthetic reactions. In this connection, the experiments of Loomis & Lipmann (179), Cross *et al.* (68), and Clowes *et al.* (55) with cell-free preparations suggested that there was an uncoupling of phosphorylation from oxidation, but this view is now challenged by the work of Teply (287).

When the concentration of nitro-phenols is increased, then both respiration and synthetic processes are inhibited. However, Krah1 *et al.* (166) report that the activity of both a number of metal-containing oxidising enzymes and dehydrogenases is not affected by a thousandfold increase above the concentrations of nitro-phenols which are physiologically active. On the other hand, these workers also found that two typical flavoprotein catalysts, *D*-amino acid oxidase and heart muscle flavoprotein, were inhibited by $10^{-3}M$ 2,4,5-trichloro-phenol at pH 8.3 and also by 3,5-dinitro-*o*-cresol and 2,4-dinitro-*o*-cyclohexylphenol. This work was followed up by Haas and his colleagues (117) who demonstrated that in a system oxidising glucose-6-phosphate through coenzyme II and cytochrome-*c*, two enzymes, the dehydrogenase *Zwischenferment* and cytochrome reductase (a flavoprotein), were inhibited by $10^{-3}M$ 2,4-dinitro-*o*-cyclohexylphenol; on the other hand, cytochrome oxidase was not sensitive to this inhibitor. This particular system plays only a very small part in the respiration of yeast [Work & Work (319)], while little is known of the importance of flavoprotein enzymes in higher plants.

The concentrations of 3,5-dinitro-*o*-cresol required to inhibit respiration are low. Work at Oxford shows that the concentrations needed to halve the respiration rate of discs of mustard leaves infiltrated under vacuum with buffered solutions are of the order of $1.5 \times 10^{-5}M$ at pH 4 and 5×10^{-5} at pH 6. When dinitro-*o*-cresol is sprayed on to mustard seedlings, the material penetrating into the cells is likely to operate at a pH governed by the buffer systems of the tissues and, assuming a slightly acid value, it can be taken that the respiration rate is halved at approximately $3 \times 10^{-5}M$. On the other hand, the concentration of dinitro-*o*-cresol in a spray solution required to produce a 50 per cent kill ranges from $2.5 \times 10^{-3}M$ for a highly susceptible species, like mustard, to a concentration ten times as great for less susceptible species.

It is therefore evident that there is a considerable divergence between the concentration needed to interfere with respiration at the cell level and the

external concentration required to bring about death. Undoubtedly, during entry through the cuticle or stomata [Fogg (96)] and the subsequent passage from cell to cell much dilution will take place, but nevertheless it seems probable that some of the cells may well be subject to concentrations which cause precipitation of cell proteins. The use of picric acid as a fixative at strengths of approximately $5 \times 10^{-2} M$ is well known and it has been recorded by Bancroft & Richter (16) for phenol and by Stellwaag & Staudenmayer (272) for dinitro-*o*-cresol that in microorganisms proteins are precipitated at $10^{-1} M$. Moreover, Hotchkiss (140) and Gale & Taylor (103) have found that phenol, cresols, and *o*-chloro-phenol at comparable concentrations cause in bacteria and yeasts a leakage of cell constituents into the external medium.

Relationship between chemical structure and toxicity.—In a review of the literature up to 1927, Tattersfield (279) showed that the toxicity of phenol or benzene to insects and fungi is increased by the substitution of a nitro group in the *ortho* or *para* position and further increased by a second nitration to give *m*-dinitro-benzene or 2,4-dinitro-phenol. A third nitration resulted in a fall in toxicity. These conclusions are now confirmed by later work with fungi, bacteria, and Echinoderm eggs [Cavill *et al.* (50), Cowles & Klotz (60), and Krahl & Clowes (164)]. Brief reports from Crafts (61) and Chabrolin (51) also suggest that comparable relationships hold for higher plants.

Lengthening of an alkyl side chain up to a varying number of carbon atoms frequently increases the toxicity of the molecule. Kagy (151), investigating the homologous series 2,4-dinitro-6-alkyl-phenol and using the larvae of the silkworm (*Bombyx mori*) as a test organism, found that the toxicity increased up to the hexyl or heptyl, while the octyl compound was slightly less toxic. Crafts (61) has claimed in tests on unspecified higher plants that "the 2,4-dinitro compounds of phenol, *o*-methyl, *o*-ethyl, *o*-isopropyl and *o*-secondary butyl phenol, respectively, increase in toxicity in the order named. The *o*-amyl³ substituted compound drops off in toxicity." It is doubtful whether it is legitimate to arrange these particular compounds in one series since this leaves out of account any changes in toxicity that may result from a substitution of a straight chain for a branched side chain. Investigations at Oxford (28) employing the following tests (*a*) depression in respiration of yeast, (*b*) reduction in the frond multiplication rate of *Lemna minor*, and (*c*) the killing of mustard seedlings, demonstrate that 2,4-dinitro-phenol is less toxic than the methyl homologue and that there are no large differences between the remaining series up to the octyl. Spraying experiments both in the field and in pots by Blackman *et al.* (31), Roberts (238), and Roberts & Blackman (239) have demonstrated that in terms of percentage kill 2,4-dinitro-6-secondary-butyl-phenol is one to four times as toxic as 3,5-dinitro-*o*-cresol, the variation being dependent on the species of annual weed. For some species like the pea there is evidence [Blackman (27)] that the order of toxicity between these two compounds is reversed.

³ Only the *o*-secondary-amyl substitution is referred to in the rest of the paper.

Dinitro-phenols as herbicides.—Dinitro-phenol and 3,5-dinitro-*o*-cresol were first developed as selective herbicides for the control of annual weeds in cereal crops. Schwendiman *et al.* (252) in 1943 observed that some leguminous crops were more resistant than others when treated with sodium dinitro-*o*-cresylate, but more recent developments have been concerned with the use of 2,4-dinitro-6-secondary butyl and amyl phenols principally as the ammonium salts. Osvald & Åberg (220) record that legumes vary in their susceptibility to ammonium dinitro-secondary-butyl-phenate. Similarly, Roberts & Blackman (239) have found that in the 13 species tested, *Vicia sativa* and *Trifolium dubium* are the most resistant and *Anthyllis vulneraria* the most susceptible, while there was some indication that injury in some species could be correlated with morphological characteristics.

With these secondary alkyl phenols, effective weed control in peas has recently been reported in the United States by Dearborn (72) and for peas and lucerne good results have been obtained by Jägerstahl (147) in Sweden and by Roberts & Blackman (239) in England.

Warren & Buchholtz (303), discussing the variable effects under American conditions of sodium dinitro-*o*-cresylate on peas, point out that different weather conditions might, in part, account for the differences in resistance recorded by various workers. Dearborn (72) has observed that peas are more liable to injury by ammonium dinitro-secondary-butyl-phenate when the temperature is high (80° F.) than when it is relatively low (50° F.). Woodford (318) cites results which indicate that the temperature factor is more important for dinitro-6-butyl-phenol than it is for dinitro-*o*-cresol.

Apart from leguminous crops, Ryker (246) reports initial experiments with the ammonium salts of the amyl and butyl compounds for the eradication of weeds in rice, while Meenen & Timmons (193) have used these compounds in buffalo grass (*Buchloë dactyloides*) grown for seed. These phenols, mixed with diesel oil, have been employed by Hansen *et al.* (124) for the eradication of cereal windbreak rows in vegetable crops and for the control of *Sclerotinia vaccinii* and weeds in blueberries [Johnston (150)].

In addition, further investigations on the value of dinitro compounds as pre-emergence sprays have been undertaken. Both ammonium dinitro-secondary-butyl-phenate [Scarborough (249)] and dinitro-secondary-butyl and secondary-amyl phenols [Creasy & Cowart (66)] appear promising for the treatment of cotton seedbeds, while encouraging results have been obtained by Lachman (169) with the butyl phenol for the pre-emergence spraying of corn.

OILS AND HYDROCARBONS

In recent years, as a result of trial and error, many applications have been found for the use of mineral oil fractions, both as general herbicides and for the selective control of weeds in a variety of crops. Since very little is yet known of the physiological or metabolic factors involved, this review will be mainly concerned with the interrelationship between variations in phytotoxicity and the chemical and physical properties.

Relation between toxicity and physical and chemical properties of oils.—The primary separation of a crude mineral oil into fractions is on the basis of boiling point and there is a broad relationship between the boiling range of the fraction and the phytotoxic effects. According to Crafts & Reiber (65), the most volatile fraction normally produced (boiling range 70 to 200°C.) causes rapid injury to the leaves of many species. There is evidence that within this fraction the less volatile components are the more toxic, possibly because the lighter constituents evaporate from the leaf surfaces so rapidly that there is little penetration. Somewhat heavier oils of the kerosene type (boiling range 150 to 300°C.) also produce rapid injury to foliage. A distinction can again be made between the components in this fraction, for Moore & Graham (202) observed that the heavier components were toxic to tomatoes, while Lachman (168) has found that they were more effective for the control of some weed species. There is also a difference in the nature of the injurious effects, since the less volatile sub-fraction brings about a much slower discoloration of the leaves [Crafts & Reiber (65)]. The oils now used for selective weed control are produced mainly from the upper part of the gasoline range and from the kerosene fractions [Bell & Norem (19)]. Gas and diesel oils (boiling range circa 280 to 380°C.) are in general more toxic than the lighter products, the toxic effects develop more slowly, and they have no selective action [Crafts & Reiber (65)]. These oils are therefore primarily used as general herbicides.

Gas and diesel oil can again be divided on the basis of boiling point, for the toxicity tends to increase as the boiling point rises [Crafts & Reiber (64)]. Lubricating oils of still higher boiling range are little used as herbicides but are extensively employed as insecticidal emulsions for fruit trees [Cunningham & Muggeridge (69) and Cole (57)]. Only the lighter of these oils are suitable for use as foliage sprays, but heavier lubricating oils can be applied to fruit trees in the dormant phase [Marshall (189)].

Although there is a broad linkage between boiling point and phytotoxicity in the several fractions from any one crude oil, the assumption cannot be made that fractions of a similar boiling range refined from crude oils of different oil fields will bring about equitoxic effects. Some of this divergence is due to variations in the content of "total aromatics," which in most unblended oils is roughly equivalent to the proportion which can be sulphonated. The aromatic content in turn is dependent upon the type of base of the original crude oil and the refining processes undergone by the fraction [Birch (23)].

The influence of refining on toxicity has been reported by Gray & de Ong (109) who stated that products of high sulphonatable content are the most toxic to plants. Again, according to Allen & Carpenter (4), fractions derived from "naphthenic crudes" are more toxic than fractions refined from paraffinic crude oils. The correlation between aromatic content and toxicity is relatively high when a fraction from a single source is subject to various degrees of refining [de Ong *et al.* (217)], but in comparing different fractions

or products from different sources, Green (110) emphasises that aromatic content alone is no longer a reliable guide to the phytotoxic effects, for highly refined oils with a very low aromatic content can be toxic. Moreover, Crafts & Reiber (65) have shown that unsulphonatable residues of gasoline and the lighter stove-oil fractions are often acutely toxic.

On the basis of his investigations, Green (110) concluded that the criteria of density, viscosity, surface tension, and flash point are of little value in assessing toxicity, while such properties as bromine absorption, iodine value, aniline value, and refractive index are related to boiling point and aromatic content rather than to toxicity [Tutin (296), Bell & Norem (19)].

Phytotoxicity of hydrocarbons.—A different approach to the understanding of the herbicidal action of mineral oils is through the study of the varying toxicity of the hydrocarbons of which oils are composed. Lachman (168) observed that a number of aromatic compounds with boiling points within the range of "Stoddard solvent" type oils were also selectively toxic to weeds in carrots when they were applied in a carrier of paraffinic kerosene. Crafts & Reiber (65) tested a number of hydrocarbons diluted with a highly refined kerosene. In the aromatic series they studied, toxicity tended to increase both with the number of substitutions in the benzene ring and with the size of the substituted groups. The naphthenes investigated were of a comparable toxicity to the aromatic compounds and cyclohexane was more toxic than either methyl-cyclopentane or methyl-cyclohexane. On the other hand, even when the paraffins *n*-hexane, *iso*-octane and *n*-hexadecane, were applied undiluted, they were only slightly toxic.

There are several disadvantages in using carriers. Firstly, no satisfactory diluent which is completely nontoxic has been found. Secondly, carriers tend to mask differences in the physical characteristics of the compounds under investigation. For example, Leonard & Harris (174) found that when a highly refined kerosene was substituted for hexane as a diluent, the toxicity of some aromatic compounds was increased.

The most comprehensive survey of the effects of undiluted hydrocarbons has been carried out by Havis (128). In each of the main groups the most volatile compound was the least toxic. Aromatic compounds were more toxic than olefines of comparable boiling point and these in turn were more injurious than paraffins, while naphthalene derivatives were the most toxic.

At present, the investigations with hydrocarbons have not proceeded far enough to allow generalisation. With some series there is evidence that toxicity increases with the number of carbon atoms. Though between the three groups, aromatic compounds, olefines, and paraffins, there is a descending order of toxicity, there are many exceptions. Paraffins such as *n*-decane have a relatively high toxicity [Young (325)]. Of the few naphthenes tested some, such as decahydro-naphthalene and dicyclohexyl, are also highly toxic. Since oils of the white spirit type used as selective herbicides contain some of the more toxic compounds of each class, e.g., such active compounds as *n*-decane, decahydro-naphthalene, and diethyl benzene

[Sachanen (248)], it is evident that the specifications for fractions which suffice in oil technology are biologically inadequate.

Oils as herbicides.—Following the successful use of oils for selective weed control in umbelliferous crops [Norman *et al.* (209)] their application has been extended to the eradication of weeds in the nursery seedbeds of conifers [Stoeckeler (275), Eliason (84)]. Emulsions of light oils of a high aromatic content are being employed in the United States for the control of weeds in irrigation channels, etc. (9). Both Herbert (133) and Evans (91) report that certain light oils have killed weeds in oil flax without an undue check to the crop, while Grigsby (114) states that *Digitaria sanguinalis* can be differentially killed in lawns. A novel method of weed control has been worked out by Talley (278) taking advantage of the observation that the basal stem of cotton bears a waxy covering which apparently protects the internal tissues from the oil. In consequence, by using a special sprayer such that only the basal stems of the crop plant come in contact with the spray droplets, the weeds can be selectively killed. It is essential to use minimum volumes and the content of both the aromatic and naphthenic compounds is important.

MISCELLANEOUS HERBICIDES

Although the main stream of research has been concerned with plant growth regulators, aryl nitro-compounds, mineral oils, and hydrocarbons, a wide range of chemically divergent compounds has also been examined for herbicidal value. The investigations have been confined to field trials and there is as yet scant information on their mode of action or the physiological and metabolic effects brought about by treatment.

Pentachlorophenol and its sodium salt, which are somewhat toxic to insects and mammals, can be absorbed through the skin and have some cumulative action [Kehoe *et al.* (153), McGavack *et al.* (188)]. Alban & McCombs (2) report that these compounds show considerable promise for the pre-emergence spraying of seed beds sown with such crops as snap and lima beans, onions, spinach, sweet corn, radish, tomato, and asparagus. The persistence of the sodium salt in the soil has been examined by Loustalot & Ferrer (180) who found that after storage for two months at 10°C. the treated soil was still toxic to maize and cucumber, but that at higher temperatures the rate of breakdown was accelerated. Pentachlorophenol continues to be used as a fortifying agent in oil emulsions and with the further addition of butyl 2,4-D has been employed for the control of weeds in coffee plantations [Smith *et al.* (267)]. Other uses have been summarised by Sherwood (259).

Potassium cyanate, according to Wilson & Easley (314), possesses a low toxicity to mammals but is differentially toxic to plants. It has given satisfactory results for the control of weeds in onions [Lachman (171)], in asparagus [(LeCompte (172))], and for the eradication of *Digitaria sanguinalis* from turf [Nutter & Cornman (212) and Walter & Wolf (302)]. Under conditions of drought and low temperatures, its efficiency is reduced.

Inorganic salts of trichloroacetic acid are particularly toxic to some species of grasses. On the basis of Sterling's (274) account of the various trials carried out in the United States and Canada, it can be concluded that a high degree of control of *Agropyron repens* can be obtained when 100 pounds or more is applied per acre. There is some indication [McCall & Zahnley (187)] that for a given quantity a divided application is more effective. For the control of *Sorghum halepense*, which has a deep root system, spraying the foliage is more effective but with shallow rooted species, such as *A. repens*, treatment of the soil is better. Carter *et al.* (49) have killed *Cynodon dactylon* in peach orchards while a partial control of *S. halepense* in sugarcane stubble has been reported by Hagood (119). For the control of *Cyperus rotundus* trichloroacetates are ineffective (59), but *Opuntia humifusa* is susceptible (187).

According to Loustalot & Ferrer (181), the breakdown of trichloroacetic acid in soils is accelerated by high temperatures and high moisture content. These workers also observed that persistence was greater in clay soils, but Arakeri & Dunham (11) did not find that the soil type was important. The overall period of persistence also differed in the two investigations, but these divergences may be due to variations in the susceptibility of the different species used as test plants in the two investigations.

Further experiments on ammonium sulphamate by Bull & Campbell (42) confirm that this compound is particularly toxic to some woody perennial species. Read (235) reports that when the material is applied to a cut in the trunk, then the effectiveness is linked with the time of day of the application. Leonard & Harris (175) state that *C. rotundus* is killed by methyl bromide, while Bruner (38) concludes that ethyl xanthogen disulphide is suitable for the pre-emergence treatment of a variety of crops.

PART II

PLANT GROWTH REGULATORS

Since the previous review by Norman *et al.* (209), the nature of the toxic action of plant growth regulators has been little elucidated. The papers that have been published emphasise the wide diversity of the effects produced and the present account necessarily relates only to research which is considered to have a direct bearing on the problems of herbicidal action.

PHENOXYACETIC ACID DERIVATIVES: BIOCHEMICAL AND PHYSIOLOGICAL EFFECTS

This group includes 2-methyl-4-chlorophenoxyacetic acid (MCPA, MCP or Methoxone), 2,4-dichlorophenoxyacetic acid (2,4-D or DCPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T or TCPA).

Relationship between chemical structure and activity.—The relationship between chemical structure and activity has been reviewed by Went (308),

Wain (301), and Sexton (254), while Veldstra & Booij (299) have added to Veldstra's earlier contributions (298). It seems evident that relative activity is to some extent dependent on the nature of the physiological test employed. Muir *et al.* (203) have indicated that 2,4-D and indole acetic acid possess similar growth activity as measured by cell elongation. Kalinowski & Kalinowski (152) have shown that 4-chlorophenoxyacetic acid, which is active, can be cyclised to 5-chlorocoumar-3-one, which is inactive. Audus (14), studying root growth inhibition by 2,4-D and coumarin has concluded that only the undissociated molecule is active.

Penetration.—Hamner & Chi-Kien (123) claim that the inclusion of plastic material in 2,4-D sprays increases herbicidal action by sealing in toxic vapours, but probably, as Henderson (131) suggests, owing to the low vapour pressure of 2,4-D, the effect of the plastic is to reduce local evaporation and thus allow more effective penetration from the spray droplets. Mitchell & Linder (198) using radioactive iodine (I^{131}) in 2,4-dichloro-5-iodo-phenoxyacetic acid were able to show that very young and fully expanded leaves could not absorb this compound as well as partially or only recently expanded ones; that young but fully expanded leaves under favourable conditions had a relatively constant absorption rate; that the radioactive material when applied to the leaves accumulated mainly in the upper part of the stems; and that wetting agents accelerated absorption.

Translocation.—Translocation of 2,4-D from the primary leaf of a bean seedling to other parts of the plant was observed by Weintraub *et al.* (306). Wood *et al.* (316) found that 2-iodo-3-nitro benzoic acid containing I^{131} was translocated from treated bean leaves to the stems where it accumulated mainly in the terminal buds and hypocotyls. In barley seedlings, accumulation was mainly in the second leaf. Passage of 2,4-D activity from stock to scion was recorded by Dhillon & Lucas (74). Tullis & Davis (295) found that *Stillingia celifera* injured by 2,4-D in the summer of 1948 still showed symptoms of injury on the earliest growth in the following spring, but this did not occur with *Melia azedarach*. Wurgler (322) concludes that upward movement in woody plants is more rapid than downward.

Rohrbaugh & Rice (241) considered that in the dark 2,4-D was not translocated from starch-free bean leaves unless sugar was added to the leaves. In this respect fructose and glucose were more effective than sucrose. Similar effects have been recorded by Weintraub & Brown (305) who concluded that there was no apparent combination between the sugar and the growth regulator. Translocation, facilitated by sugars, normally takes place only in living cells (presumably in the phloem) but may occur in nonliving tissues independently of sugar movement when the growth regulator is introduced into the transpiration stream. Davis & Smith (71) found that after 36 hr. in the dark, the carbohydrate content was reduced to a level at which 2,4-D was ineffective unless applied through the cut end of a petiole or a "sliver" of hypocotyl, while mixtures of 2,4-D with sugars (particularly

sucrose) remained lethal. The respiration of starved plants was stimulated by this growth regulator and semi-permeability was apparently destroyed, thereby allowing exudation of cell sap and tissue collapse.

Environmental factors and phytotoxicity.—Marth & Davis (190) have shown that phytotoxicity increases with temperature, a result confirmed by Kelly (155); but according to Penfound & Minyard (227) 2,4-D butyl ester in kerosene was more toxic to plants grown in the shade. Other factors have been reviewed by Hamner (122). Variations in response to MCPA sodium salt according to the time of day when the material was applied were observed by Greenham (112), under certain conditions, in bean plants, but not in *Chondrilla juncea*.

Chemical changes within the plant.—Wort (320) recorded increases in the nitrogen content of stems and roots of 2,4-D-treated buckwheat, becoming greatest after four to eight days. Total sugars and starch-dextrins approached a maximum in the stems at the end of two days, while the nitrogen content of the leaves steadily declined. Epinasty was followed by increased stem turgor and ease of snapping suggested a change in character of the cell walls. Necrosis of the first internode prevented upward translocation and longitudinal splitting hastened desiccation of the stems. Weller (307) observed reduced percentages of protein, certain amino acids and nonreducing sugar in the leaves and roots of treated kidney beans, but no significant change in reducing sugar, starch, polysaccharides, crude fibre, total ash, ether extract, or unsaponifiable material. Sell *et al.* (253) found that protein and amino acids accumulated in greater quantities in the stems of treated kidney beans and that crude fibre and reducing and nonreducing sugars diminished while ash, ether extract, unsaponifiable material, and fatty acids were increased. It was suggested that the character of the protein was changed by treatment. These workers (184) also reported that the same treatment increased the leaf content of pantothenic acid and reduced the amounts of thiamine, riboflavin, nicotinic acid, and carotene, whilst in the stems, except for carotene which was depressed, these constituents were augmented.

Yakushkina (323) states that local application of a 2,4-D spray to the flowers of tomato doubled the sugar level but slightly depressed nitrogen and total ash. Respiration was more than doubled at first and then decreased, catalase activity rose, while that of peroxidase was halved and reverse effects took place in the leaf tissue. The total alkaloid content of *Datura stramonium* was not influenced by 2,4-D [Tsao & Youngken (293)].

Ergle & Dunlap (88) found that increasing applications of 2,4-D raised the concentrations of sucrose, hemicelluloses, and cellulose in the main stalk of cotton, while reducing sugars and total organic acids varied inversely with the amount applied. The starch content of the leaves was only affected at the highest rate. Gall (104), culturing bean stem sections on an agar-starch-2,4-D medium, found that starch degradation was greater in the absence of 2,4-D.

Amongst the effects on enzyme systems, Goldacre (108) reported that

2,4-D increased the rate of destruction of indole-3-acetic acid by a crude preparation from etiolated pea epicotyls. Kvamme *et al.* (167) showed that wheat germ lipase was inhibited and Ravazzoni (234) claimed an increased action of phosphate enzyme systems. Hagen *et al.* (118) found no inhibition of polyphenol oxidase, α -hydroxy acid oxidase, or catalase but the activity of castor oil bean lipase was depressed. An interesting point is that the butyl ester was inactive as a lipase inhibitor and had to be first hydrolysed to the acid. Neely *et al.* (206, 207) found that 2,4-D lowered the activity of α - and β -amylase in kidney bean stems and that, in the proliferating tissues of both stems and leaves, pectin methoxylase activity was enhanced and that of phosphorylase depressed.

Helgeson *et al.* (130) record that the protein content of grain from bread wheats was slightly increased by 2,4-D esters, while the protein content of durum wheat was decreased by the amine and sodium salts. Similar increases in the protein content of treated wheat have been observed by other workers (North Central Weed Control Conference Research Report, 1949). No differences in milling or baking quality were recorded by Buré (43) or Friesen (101).

Mineral uptake.—Studying the reaction of plants to MCPA, Rhodes *et al.* (237) at Jealott's Hill Research Station found a marked diminution in the potassium content of the aerial portions and a marked accumulation in the roots, while the overall content was greatly lowered. Since the compound was added to the culture medium, it appears that there was a specific inhibition of potassium uptake combined with an interference either in transport to the shoots or in redistribution of potassium between root and shoot. Of the three species examined, the most susceptible to MCPA showed the greatest changes in the potassium relationships. No significant changes in nitrogen or other mineral nutrients were observed.

Effects on growth and development.—According to Greulach & Singh (113), low concentrations of 2,4-D which do not depress the total dry weight of bean plants may decrease the ratio of reproductive to vegetative parts. Similar concentrations may delay the maturity of kidney beans, increase lateral growth, and, despite deformation, cause more leaves to be produced [Stromme & Hamner (276)]. Staniforth (271) believes that the inhibitory effect may occur during meristematic differentiation rather than cellular growth.

General morphological effects have been studied by many workers, Murray & Whiting (205) with kidney beans, Wurgler (321) with *Prunus persica*, Treccani (294) with tomatoes, marrows, and watermelons, Zimmerman & Hitchcock (328) with *Kalanchoe*, Barry (17) with hemp, and Klein & Link (159) with flax. Cotton has been studied by Burton (44), Rakitin *et al.* (232), Okanenko & Tabentskli (215), and cereals by Åberg & Denward (1) and Swanson *et al.* (277).

Other effects.—Freeland (100) found that the rate of photosynthesis in *Anacharis canadensis* was depressed by 2,4-D and that at the rates used,

after an initial decrease in respiration, there was partial or complete recovery in 48 hr.

The effects of dichlorophenoxyacetic acid in tissue cultures have been recorded by Hildebrandt & Ryker (134) and Gautheret (106). Cytological studies have been continued by Ryland (247), Nygren (213), d'Amato (5), and Doxey & Rhodes (77).

Several authors [e.g. Ciferri (53) and Petersen & Thorup (228)] have drawn attention to the similarity between the effects produced by 2,4-D and the symptoms of virus, insect and fungal attack.

SUBSTITUTED PHENOXYACETIC ACIDS AS HERBICIDES

Heal & Thompson's (129) review contains many references to the value of 2,4-D as a herbicide together with selected references on MCPA, 2,4,5-T and IPPC. Lists of weeds classified according to their susceptibility to these compounds have been prepared by Beckley (18), Easterbrook (82), Holmes (139), Lynch (185), Orchard (218), Osvald & Åberg (220), Pearse (224), members of the North Central Weed Control Conference (162, 194, 223), and many others.

Post-emergence application: weed control in arable crops.—From an economic viewpoint the most important application of plant growth regulators for weed control has been in cereal crops. Templeman & Halliday (282), for example, have estimated the average potential increase in grain yield resulting from weed eradication at approximately 350 lbs. per acre. Similarly, on the basis of some 139 experiments in Germany, Wiede (309) has calculated that the gain in yield resulting from 2,4-D application was 550 lbs. per acre or over 21 per cent. The level of increasing productivity following on weed eradication is, however, dependent upon two factors: the effect of the removal of weed competition and the direct effect of the treatment on the crop. Under some circumstances it has been shown by experiments on clean crops that the yield may be depressed. Comparison between the results of different workers is rendered difficult by the possibility of significant second order interactions between variety, stage of development at the time of treatment, and the compound used. In Denmark, for example, Pedersen *et al.* (225) observed varietal differences in oats but not in barley to treatment with 2,4-D but in neither crop to treatment with MCPA.

In Belgium, Lacroix (171), Moës (200), and Noulard (211) have concluded that MCPA is less likely than 2,4-D to injure wheat, oats, and spring barley. Andersen & Hermansen (7) have found that wheat is the most resistant of the three, while the liability to damage is greatest in barley at the 2-4 leaf stage and in oats at the 3-6 leaf stage. The Swiss workers, Gallay *et al.* (105), also draw attention to the abnormal fluorescences in spring oats and barley which may occur when the plants are treated when 3 to 4 inches high. Hagsand & Väärtö (120) record that under Swedish conditions abnormal ears were sometimes formed if spraying took place within three weeks of emergence. In the United Kingdom, on the other hand, Temple-

man & Halliday (282) have shown that correctly-timed dust or spray applications of up to 8 lb. per acre MCPA sodium salt did not cause any reduction in the yield of weed-free crops of wheat, oats, or barley. The latest Jealott's Hill findings (286) indicate that MCPA butyl ester may be used with safety in spring cereals at somewhat heavier rates than the corresponding ester of 2,4-D and, further, that an oil formulation of the MCPA ester was more effective against certain weeds, including *Polygonum* spp., *Papaver rhoeas*, and *Matricaria inodora*, than an aqueous solution of the sodium salt. In France, Longchamp & Gautheret (177, 178) noted that the yield of "Versaille" oats was unaffected by applications of up to 7 lb. per acre 2,4-D, though yields of wheat and barley were seriously reduced by smaller dressings. Although the use of MCPA or 2,4-D at rates up to 2 lb. per acre, as the sodium salt, is generally recommended in Great Britain [e.g., Blackman & Holly (29)], Templeman & Halliday (281) have shown that, at this rate, MCPA gives excellent control of annual weeds such as *Sinapis arvensis*, *Thlaspi arvense*, *Chenopodium album*, and *Raphanus raphanistrum*. Kole (162) in the Netherlands prefers to use these compounds only against weeds resistant to dinitro-*o*-cresol and calcium cyanamide. Work in the North Central United States has been reviewed by Phillips (229) and Olson (216), where it is generally agreed that autumn sown cereals are liable to damage by autumn applications of 2,4-D. There is some difference of opinion regarding the effect of spring applications and varietal differences in the sensitivity of spring cereals have been observed. In one experiment, Buchholtz (41) noted that the yield of spring oats treated with MCPA was significantly greater than when corresponding rates of 2,4-D were applied.

Discussing results on maize (corn), Lee (173) has reported that this crop is least tolerant to 2,4-D at a height of 10 to 36 inches and most tolerant at 2 to 4 inches, hybrid varieties being the most sensitive. Rossman & Staniforth (244) found differences in resistance between four inbred lines, but all were seriously injured when treated either at the 6-8 leaf stage or at tasselling. Subsequently, Rossman & Sprague (243) found that in some instances the yield of single cross seed from these treated inlines was depressed. From the investigations of Lachman (169), Dearborn (72), and Bender (20) on sweet corn, it can be concluded that 2,4-D can be used for weed control up to 0.75 lb. per acre, irrespective of formulation.

Similarly, in a recent summary Elder (83) concluded that 2,4-D at 0.5 lb. per acre could be safely employed on most varieties of grain sorghum. Successful control of a number of important weeds in rice with MCPA and 2,4-D has been reported from Italy (52, 94, 230), Malaya (148), and the United States (245). Yields were not depressed provided rates did not exceed 0.5 to 1.5 lb. per acre and treatment took place at least 3 to 5 weeks after emergence but not after heading.

The use of phenoxyacetic acid derivatives for weed control in sugar cane, either alone or in conjunction with pentachlorophenol and other herbicides, is now well established and accounts have been given by several investigators

(63, 90, 35). The theory has been put forward (8) that, apart from weed elimination, the herbicidal treatment may raise the sugar content of the cane.

The effects of MCPA and 2,4-D on flax have been studied in some detail by Blackman *et al.* (27, 31) who concluded that MCPA was safer to use on this crop than 2,4-D, that concentrations which did not affect seed production might yet depress fibre production, that ester oil emulsions were highly injurious, and that, in general, the young seedling phase was the most resistant. Resistance and susceptibility were shown to be inheritable. The investigations of Valle *et al.* (297), Paatela & Dunham (222), Hagsand & Väärtnöu (120), and Jacobsen (146) are in general agreement with these findings. Reviewing research in the North Central United States, Robinson (240) concluded that ester formulations of 2,4-D were the most injurious, the sodium salt the least harmful, and amines intermediate.

Weed control in grassland.—Halliday & Templeman (121) have described experiments in which some of the worst weeds of British grassland, e.g., *Cirsium arvense*, *Senecio jacobaea*, and *Ranunculus* spp. were controlled by suitably-timed MCPA applications. In comparison with 2,4-D, MCPA appeared to be more effective against *Ranunculus* spp.—except *R. bulbosus*—but slightly less effective against *S. jacobaea*. *Rumex* spp. were controlled only in the seedling stage. Blackman (26) has emphasised that in some species, e.g., *Taraxacum officinale*, the relative toxicity of these compounds may be reversed between spring and autumn applications. Clovers are sometimes partially suppressed. Buchholtz (40) suggests that MCPA may be less toxic than 2,4-D to red clover, alsike, and sweet clover. Lynch (186) has demonstrated that in New Zealand *S. jacobaea* may be controlled by repeated treatment over a period of several months. Although most *Rumex* spp. appear to be resistant once they have passed the seedling stage, Scarponi (250) reports that older plants of *R. crispus* are susceptible.

The successful control of weeds in grasses grown for seed production has been described by Templeman (280), Hagsand & Väärtnöu (120), and others. Cocksfoot (*Dactylis glomerata*) and bent (*Agrostis* spp.) appear to derive less benefit from weed control treatment with 2,4-D than other grasses such as timothy, fescue, ryegrass, and meadow grass (3, 160, 191).

Weed control in horticultural crops.—Many horticultural crops including the grapevine are too sensitive to the substituted phenoxyacetic acids for selective weed control to be practised. In the case of strawberries, weed control with these plant growth regulators is feasible but the stage of development and especially the tolerance of the variety must be taken into account (46, 183, 300). Hylmö (142) has successfully used MCPA for selective weed control in peas, and Blackman (25) and Grigsby *et al.* (115) have shown that perennial weeds such as *Cirsium arvense* and *Convolvulus* spp. can be kept down in asparagus beds by spot treatment.

Pre-emergence application.—Interest in the pre-emergence application of phenoxyacetic acid derivatives, first reported in 1945 (263), has recently

centred upon crops liable to damage by post-emergence applications at rates which are high enough to control annual weeds. Reviewing American work on maize, Fuelleman (102) concluded that effective and selective weed control only resulted when the soil was moist enough for both weeds and crop to germinate. Willard (311) considered that pre-emergence treatment could be safely used on field and soya beans, provided no rain fell between the application of the herbicide and emergence of the beans. According to Templeman & Wright (285), pre-sowing applications which prevented the emergence of a range of weed species were without effect on kale, mangolds, lettuce, onions, beans, peas, lucerne, and sugar beet, provided that the dosage did not exceed 1 lb. per acre and the application was made at least three to four weeks before sowing. Pre-emergence treatment in sugar beet, when applied after sowing, has generally given poor results [Willard (310)]. Andersen & Shadbolt (6) found that potato plants were temporarily distorted by 2,4-D applied at 1 to 2 lb. per acre just before emergence, but that excellent weed control was obtained and yields were not reduced. Similar results were obtained by Smith, Marshall & Meadows (265). Experiments on peas, flax, sunflowers, castor beans, and other crops have been summarised by Slife (264). In general, the best results were obtained if treatment was delayed four to seven days after sowing and the soil was moist at the time the herbicide was applied. Promising results have been obtained with gladiolus [Jenkins (149)].

PHENOXYACETIC ACID DERIVATIVES AND CONTROL OF PERENNIAL WEEDS

Herbaceous species in arable land.—The use of plant growth regulators for the control of annual weeds is now well established and attention has been directed towards some of the more persistent herbaceous perennials. Reviews of American results on *Cirsium arvense*, *Sonchus arvensis*, *Lepidium draba*, *Convolvulus arvensis*, *Euphorbia esula*, and *Centaurea repens* are contained in the Research Report of the 1949 North Central Weed Control Conference. The success of herbicidal treatment is clearly dependent not only on the dosage and formulation but also on correct timing. Blackman & Holly (30), for example, have found that MCPA sodium salt is most effective against *Lepidium draba* when the flowerbuds are forming, whereas 2,4-D is best applied when the plants are already in full flower. Similarly, optimum control of *Cirsium arvense* is obtained when treatment is delayed until the pre-flowering stage, whereas with *Ranunculus repens* good control may be obtained at any time during the period of active growth and with *Senecio jacobaea* the seedling stage may be the most susceptible (26). Control of *Equisetum arvense* by repeated applications of MCPA has been reported by Jackman & Tincker (145).

The use of phenoxyacetic acids for the control of ditchbank and roadside weeds has been described by Hilli (136), Denward (73), and others. Further American investigations on the eradication of *Eichhornia crassipes* and other aquatic weeds have been reported by Hitchcock *et al.* (137) and Zimmerman

et al. (329), while Thomas & Srinivasan (289) have summarised results in India. Control of parasitic plants such as *Orobanch*e and *Loranthus* has been described by Ovcharov (221) and Hartigan (127). The effect of 2,4-D on *Cyperus rotundus* has been studied by Eames (80) and Loustalot & Delgado (182).

Woody species.—2,4,5-T has been shown to be generally the most effective of the phenoxyacetic acid derivatives against woody species. Offord & Moss (214) killed *Ribes binominatum* with 2,4,5-T at 250 p.p.m. and other *Ribes* spp. at 2,000 p.p.m., concentrations at which 2,4-D was ineffective. Control of *Rubus* and other species has been reported by Orchard (219) and Green (111), of *Lantana camara* by Easterbrook (81) and du Toit (292), and of *Corylus* spp. by Zehngraff & von Bargen (327). Young & Fisher (324) have studied the effectiveness of 2,4,5,-T formulations with and without 2,4-D against *Prosopis juliflora*, and Thimann (288) has described work against *Dichrostachys nutans* in Cuba. Brush control in Texas has been reviewed by Young *et al.* (326), and Melander (194) has summarised contributions to the North Central Weed Control Conference. A few woody plants, including *Symphoricarpos* spp., are more sensitive to 2,4-D than to 2,4,5-T. Control of *Alnus*, *Betula*, *Populus*, *Pyrus*, and *Salix* spp. up to 9 ft. high with two applications of 2,4-D ester was reported by Mukula (204) from Finland.

PHENOXYACETIC ACID: PERSISTENCE AND INACTIVATION IN SOIL

Evidence on the persistence of herbicides in soil has been reviewed by Norman & Newman (210), who conclude that persistence is greatest under arid conditions when the factor of leaching is inoperative and the activities of microorganisms are checked; conversely, persistence is least where both rainfall and temperature are high, or where irrigation is employed [Erickson & Gault (89)]. Inconsistencies may occur where decomposition is retarded by the comparative absence of the particular organism needed for the initial oxidation. Audus (15), however, suggests that although inactivation is due almost entirely to microorganisms, the process does not involve oxidation. This worker has also found indications that the decomposition products may include a root growth stimulant

Meadows & Smith (192) conclude that organic matter in soil is a more important factor influencing decomposition of 2,4-D than pH, temperature, or initial rate of application. Henriet (132) records that for 2,4-D in newly limed soils the period of persistence may be 18 weeks while in medium silts the period may be four weeks. Persistence is greatest in dry clay soils. Studies on the protection of susceptible crops by "contratoxication" of 2,4-D by activated carbon have been continued by Weaver (304) and others (12, 48).

PHENOXYACETIC ACID DERIVATIVES: TOXICOLOGICAL STUDIES

The use of phenoxyacetic acid derivatives for pasture weed control has naturally raised the question of possible toxicity to livestock. Tests on labora-

tory animals by Bucher (39), Bjorn & Northen (24), Mitchell & Marth (199), and Hill & Carlisle (135) have indicated that toxicity is low and that the maximum tolerable dose of 2,4-D is of the order of 200 mg. per kg. of body weight. Effects on the nervous system of rats have been studied by Eyzaguirre *et al.* (92). Mitchell *et al.* (197) and Halliday & Templeman (121) have described experiments in which sheep and cows, receiving 2,4-D or MCPA in quantities unlikely to be exceeded in practice, were quite unharmed. None of these workers detected any taint in cows' milk attributable to the use of herbicides. Grigsby & Farwell (116) observed no ill effects on stock grazing herbage which had been freshly sprayed with 2,4-D sodium or alkanolamine salts at 4 lb. acid equivalent per acre.

The toxicity of 2,4-D to fish has been measured by Harrison & Rees (126), who estimated that the upper safe limit for sunfish and catfish was 500 p.p.m. and for minnows 1,500 p.p.m., and by King & Penfound (156), who concluded that at 100 p.p.m. 2,4-D was only slightly toxic to bream and bass but that, under certain conditions, there was a danger of killed and decaying vegetation severely restricting the supply of dissolved oxygen.

ISOPROPYL PHENYLCARBAMATE

The selective herbicidal properties of isopropyl phenylcarbamate (IPPC or IPC) were first demonstrated by Templeman & Sexton (256, 283, 284) who used this compound for pre-emergence and selective control of graminaceous weeds in dicotyledonous crops.

Physiological and cytological effects.—Alkyl phenylcarbamates are known to be narcotic poisons which interfere with cell division and the cytological effects of such compounds on a variety of plants have been studied by Hlose & Ravault (176), Doxey (76), Ennis (86), and Ivens & Blackman (143, 144). The latter workers have concluded that such cytological aberrations cannot be regarded as the primary toxic effect and that root injury may occur at concentrations which do not cause abnormal cell division. Thompson (290) was unable to find any significant changes in the carotene content, green colour, dry matter, or total yield of lucerne sprayed with IPPC.

According to Mentzer & Molho (195), isopropyl phenylthiocarbamate at half the concentration of the corresponding phenylcarbamate produces similar morphogenetic effects in wheat seedlings. The relative phytotoxicity of a series of alkyl esters of *n*-phenylcarbamic acid has been assessed by Freed (98) who claims that comparative toxicity is correlated with a function obtained by multiplying molecular refractivity by the melting point of the ester divided by the boiling point of the parent alcohol.

IPPC as a herbicide.—Templeman & Wright (285) have shown that applications of IPPC at 5 lb. per acre up to two weeks before the sowing of the crop effectively controlled grasses and *Polygonum* spp. without injury to kale, mangolds, lettuce, onions, beans, peas, lucerne, sugar beet, or swedes. By adding MCPA or 2,4-D at 1 lb. per acre, it was possible to control a wider range of weeds without harm to the crop, provided applications were

made at least three to four weeks before sowing. Against *Stellaria media* or *Galium aparine* the mixture was better than either component alone. Pre-emergence treatment with IPPC/2,4-D mixtures applied one to two weeks after planting potatoes was found by Smith *et al.* (266) to provide moderate control of dicotyledonous weeds and grasses and to increase significantly the yield of tubers. Tests with IPPC against established plants of *Agropyron repens* by Freed & Bierman (97, 99), Raleigh (233), Grigsby *et al.* (115), and Snyder (268) have met with only limited success. Carlson & Moulton (47) obtained satisfactory control of *Stellaria media* at rates of 5 to 15 lb. per acre without injury to strawberries. The susceptibility of this weed to pre-emergence treatment has been noted by Lachman (170).

Selective control of undesirable grasses in dormant lucerne has been reported by Shafer (258) and Bierman (22). Satisfactory control of annual grasses in red clover and soya beans has been described by Dutton (78) and Willard & Shaw (312). Carder (45) failed to control *Avena fatua* in oil flax with applications either before or after emergence, while the pre-emergence treatment severely damaged the crop. Such injury to flax has also been noted by Moore (207) in Australia. According to Roland (242), pre-sowing applications of IPPC of 5 to 10 lb. per acre killed selectively this weed in Swedish crops of peas, rape, and sugar beet.

OTHER SYNTHETIC PLANT GROWTH REGULATORS

The effect of maleic hydrazide (1,2-dihydropyridazine-3,6-dione) on plants was first reported by Schoene & Hoffman (251). The herbicidal properties of this compound, which shows promise both as a non-selective killer of grasses and as a selective weedkiller for the control of grasses in cotton, flax, clover, and lucerne, have been studied by Crafts *et al.* (62, 70), who found that phytotoxicity was increased by adding a wetting agent. Anthocyanin pigmentation following maleic hydrazide application was thought to result from carbohydrate accumulation in the leaves.

In greenhouse experiments, Zukel (330) killed young plants of *Digitaria sanguinalis*, *Sorghum halepense*, and *Agropyron repens* with maleic hydrazide sprayed at 0.25 per cent concentration. Older plants were prevented from seeding and root growth was checked. *Cyperus rotundus* was killed when the concentration was increased to 0.5 per cent. Temporary reduction in the growth rate of turf grasses has been reported by Engel & Ahlgren (85). Harris (125) has suggested that at low concentrations, maleic hydrazide may be used for controlling *Allium vineale* in pastures.

The *n*-aryl phthamic acids, together with their esters, amides, and soluble salts, as well as the *n*-aryl phthalimides, have been found by Hoffman & Smith (138) to possess growth regulating properties.

Tischler *et al.* (291) have claimed that 3,6-endoxotetrahydrophthalic acid, 3,6-endoxohexahydrophthalic acid, their anhydrides and salts, and other 3,6-endoxohydrophthalates may be of value as both selective and pre-emergence herbicides. Sexton, Slade & Templeman (255, 257) observed 2-

benzoyl-benzoic acid and its derivatives to display selective herbicidal action, but considered them to offer less promise than the phenoxyacetic acid derivatives for practical application. Following their earlier observation of the growth-regulating properties of 2,4-dichlorobenzylnicotinium chloride and other nicotinium compounds, Wirwille & Mitchell (315) have discovered that (4-hydroxy-5-isopropyl-2-methylphenyl) trimethyl ammonium chloride, 1-piperidinecarboxylate, and related compounds also act as growth inhibitors. Crocioni (67) has shown 5-chloro-1-methyl-2-hydroxyphenylacetic acid to be an active herbicide. Sodium 2-(2,4-dichlorophenoxy) ethyl sulphate, first reported as a pre-emergence herbicide by King & Lambrech (158), has been further studied by Gilbert & Wolf (107) and by King (157) for selective weed control in strawberries. The sodium salt of α -hydroxy- β -trichloroethylsulphonic acid ("Chlorosol A") was reported by Sterling (274) and Dutton (79) to control some grasses less effectively than trichloroacetic acid derivatives at similar rates of application, but the related compound, dichloral urea, is claimed by King (157) to be a herbicide of merit, which is both persistent and noncorrosive.

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NUTRITIONAL REQUIREMENTS OF ISOLATED PLANT TISSUES AND ORGANS¹

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The cultivation of isolated plant tissues and organs *in vitro*, that is to say the technique broadly classified as "tissue culture," represents one of the important developments in plant physiology of the past two decades. Stemming from the theoretical considerations of Haberlandt (87), it was first given a partial measure of success in the simultaneous papers of Haberlandt's student, Kotte (101, 102) and of the American botanist, Robbins (159, 160). The technique was first placed on a practical basis in the studies on cultivation of excised roots by White (201 to 204) and on cultivation of undifferentiated tissues of cambial origin by Gautheret (52 to 55), Nobécourt (134 to 137), and White (213). Since 1934 the technique has found important uses in a great variety of problems in plant physiology (202). The general literature of the subject has been reviewed repeatedly, the latest summary being that of White (222). It is the purpose of the present review to bring the subject up to date, especially as regards its nutritional aspects.

The difficulties which stood in the way of the development of a successful technique for cultivating excised plant tissues and organs between 1902 and 1934 were two: (a) the problem of choosing for study a tissue which was inherently amenable to such an approach, in which respect the stinging hairs and stomatal guard cells chosen by Haberlandt (87) had proved unsatisfactory; and (b) the development of a satisfactory nutrient. With the introduction of the excised root as a material with which to experiment (101, 102, 159), a material which proved highly satisfactory, the crucial problems became largely those of organic nutrition, a field which, even with respect to intact organisms, is still none too well understood. For convenience, the following discussion will be divided according to the nutritional factors involved.

INORGANIC NUTRITION

Major ions.—Haberlandt (87), Kotte (101, 102), and Gautheret (51) employed as a basic salt solution one or more modifications of the formula of Knop in dilute form while Robbins (159) used that of Pfeffer. White, examining the problem systematically, concluded in 1932 (201) that these solutions were not stable over a wide enough pH range to permit satisfactory study of this last factor and chose for this reason the formula of Uspenski & Uspenskaia (197). All of the ions of this solution were studied (202) and a formula arrived at which seemed satisfactory for wheat roots. Subsequent studies with tomato roots resulted in a number of modifications in the formula (208, 209, 210, 217, 218). The one finally arrived at has, since 1943

¹ This review covers the period from approximately 1939 to 1950.

(216), been standard in many plant tissue culture laboratories, although other slightly different salt solutions are recommended for root cultures by Bonner (6), Bonner & Addicott (16), and Fiedler (48). The differences do not appear to be nutritionally important—certainly excellent growth of a very wide variety of roots, embryos, and less organized plant tissues can be obtained without modification of White's formula. This formula has, in the author's laboratory, proved satisfactory for cultivation of roots of tomato, tobacco, petunia, *Datura*, egg-plant, buckwheat, radish, mustard, alfalfa, sweet clover, white clover, red clover, vetch, soy bean, carrot, sunflower, and aster [White (211)], for tumor tissues of *Nicotiana* [White (213)], sunflower [White & Braun (223)], *Vinca rosea* [White (219)], marigold and Paris daisy (White, unpublished) and for normal tissues of carrot, willow, tobacco, and *Scorzonera* (White, unpublished). It has been extensively used in other laboratories for other materials. Nevertheless, there has been entirely justified doubt of its optimal character for other tissues. Hildebrandt, Riker & Duggar (96) made a detailed study of the effects of various concentrations of all the ions represented and arrived at formulae for tumor tissues of *Nicotiana* and *Helianthus* which differed somewhat from each other and from previous formulae. Burkholder & Nickell (24) have likewise modified the formula for use with tissues of a virus tumor of *Rumex*. These differences are not crucial since some of the same tissues have been grown successfully on White's formula [White (213), White & Braun (223)].

Heller, studying normal tissues of carrot and Virginia creeper, concluded that the concentration of potassium should be increased about five fold to about 10 milligram-ions K^+ per liter (89, 90, 91). He demonstrated clear cut deficiencies for nitrogen (88, 91), phosphorus, sulfur, calcium, and magnesium (91) and showed that deficiency in nitrogen resulted in anthocyanin formation in the Virginia creeper but not in carrot (90). Riker & Gutsche made an extensive study of inorganic nitrogen sources in addition to nitrate (158), but concluded that nitrate supplied the most acceptable source of nitrogen for sunflower tissues under the conditions studied. Burström has investigated nitrate assimilation and the role of heavy metals, particularly manganese, in its catalysis (25, 26, 27, 31). He has also shown that, while phosphate functions in cell multiplication, nitrate is chiefly effective in promoting cell elongation (31). The whole question of inorganic nutrition was reexamined in 1943 by White (219).

Specific functions of these ions have not been studied in tissue culture, except for the papers of Burström mentioned above, and the experiments of Skoog (182), Skoog & Robinson (183), and Skoog & Tsui (184) who found the phosphate ion, adenine, carbohydrate, and auxin (see later) closely interrelated in the processes of control of organ formation in tobacco tissues. Phosphate is also believed to be intimately involved in carbohydrate assimilation by tomato roots [Street & Lowe (190)].

Trace elements.—Robbins, White, McClary & Bartley (171) had early shown that ash of filter paper contributed some beneficial elements for

nutrition of roots of corn not present at optimal concentrations in a Pfeffer solution plus dextrose. Nobécourt (135) introduced the use of a complex accessory salt solution developed by Berthelot (5) for bacteria, which contained cobalt, nickel, titanium, and beryllium in addition to the more usual iron, manganese, zinc, copper, and boron. This solution has also been extensively used by Gautheret (55 *et seq.*). The use of these particular elements seems to be without detailed experimental basis.

Recognizing the need for accessory elements besides the major ions, White also early examined the question, beginning with the formula of Trelease & Trelease (194) and concluded that only four trace elements were clearly significant: manganese, zinc, boron, and iodine (200). Heller, working in Gautheret's laboratory, has recently been successful in using White's formula instead of the more complex Berthelot solution (88 to 91). Eltinge & Reed verified the necessity for zinc (47). Glasstone, using a different method of estimating growth, concluded that none of these trace ions was necessary for tomato roots but that copper, in addition to iron, was essential (79). She recommends 0.01 p.p.m. Cu^{++} as CuSO_4 and 0.35 p.p.m. of Fe^{++} as FeSO_4 . In such a solution, prepared from specially purified salts, she grew tomato roots for 12 weeks without noticeable diminution in growth rate. Nevertheless her conclusions, especially of the nonessentiality of manganese (compare Burström cited above) and of zinc, are so at variance with the known requirements for intact tomato plants that most workers have continued to include the trace elements recommended by White in their nutrients.

The question has recently been reopened by Ball & Street (22). In tracing a puzzling difference in results obtained in two British cities, they came to the conclusion that if White's solution is prepared with specially purified salts, it requires copper and molybdenum, in addition to the manganese, zinc, boron, and iron usually included, in order to support the growth of excised tomato roots. The concentrations of these latter elements required (0.01 p.p.m. copper, 0.0001 p.p.m. molybdenum) however, are so low that these ions have usually, but not always, been present in the analytical grade salts used in routine work in sufficient quantities to satisfy the requirements. Ball & Street believe that differences in available quantities of these elements may be responsible for the unexplained fluctuations in weekly growth rates so frequently noted in root culture work. They point out that Glasstone's techniques were not such as would permit the detection and elimination of this level of molybdenum.

It thus appears that excised tomato roots (and probably other tissues) certainly require iron, copper, and molybdenum and probably require zinc, manganese, boron, and iodine. The results of Ball & Street strongly suggest that the list may still not be complete. These micro-element requirements have not been studied in detail for other species of roots or for other types of excised tissues, nor have the specific functions of these elements been studied in tissue cultures.

ORGANIC NUTRITION

Carbohydrates.—In the earliest studies on plant tissue cultures it was assumed that the monosaccharides, especially glucose, should be acceptable as sources of energy (Haberlandt, Kotte, Robbins, and others). This still seems to be true for tissues of monocotyledonous plants such as the maize [McClary (123)], wheat [White (201), Burström (30)] and barley first studied, but experiments with dicotyledonous tissues early made it evident that this was not always the case. White concluded in 1934 that sucrose was a more satisfactory energy source for tomato roots (204), a conclusion subsequently verified (214, 215), and concurred in by Fiedler (48) and Thielman (192). It was questioned by Robbins & Bartley (163) and Robbins & Schmidt (165) who found sucrose and dextrose of equal value and found maltose and mannose beneficial. Bonner & Addicott (16) found sucrose superior to dextrose for pea roots, Burström (32) found galactose toxic to wheat roots and Slankis found sucrose superior to other sugars for pine roots (185, 186).

A definitive conclusion on the sucrose versus dextrose question appears to have been reached as a result of the work of Street & Lowe (190) and Dormer & Street (43). These workers concluded that levulose, galactose [see also White (214, 215)], xylose, and maltose [see, however, Robbins & Schmidt (165)] are markedly toxic to tomato roots; mannose, arabinose, rhamnose, and raffinose are without nutritive value though not toxic per se; that dextrose can be utilized only to a very limited extent; and that only sucrose is a truly effective energy source. Utilization of sucrose is, as was suggested by White (221), dependent on the level of inorganic phosphate available. This agrees indirectly with the findings of Skoog *et al.* with tobacco tissue (182, 183, 184). Raffinose, which is not itself utilized, will enhance the effectiveness of sucrose, when the latter is present at low concentration, apparently by replacing in part its osmotic effects. Sucrose utilization is reversibly inhibited by phloridzin, which inhibition can be partially corrected by increase in sucrose concentration. Sucrose breakdown is quantitatively related to its utilization, one moiety of dextrose being absorbed for each two molecules of sucrose degraded. It is therefore concluded that sucrose utilization probably involves an enzymatic phosphorylation at the surface of the absorbing cells so that the material actually absorbed is a freshly formed (nascent) hexose-phosphate which cannot be replaced by the hexose and phosphate in uncombined form nor by the addition of pre-formed hexose-phosphates to the medium.

Whatever the explanation, it is now generally agreed that sucrose is the best sugar for nutrition of roots of tomato, pea, radish, clover, alfalfa, carrot, pine, and many other species, for embryos of *Datura* [Van Overbeek, Siu & Haagen-Smit (146)] and for many callus tissues [Hildebrandt, Riker & Duggar (95)]. Although Gautheret continues to use dextrose for his plant callus cultures, most if not all of his strains can be grown equally well or better on sucrose (White, unpublished). It is of interest that Slankis found sucrose to be superior to dextrose for roots of pine (186). Thus, only the

monocotyledonous plants—wheat, barley, and corn—appear to prefer dextrose or its equivalent [McClary (123), Burström (28)].

A number of studies on the effect of sugar concentrations have been made. Hildebrand, Riker & Duggar concluded that for *Nicotiana* and *Helianthus* tissues a 10 per cent concentration of sucrose was superior to the 2 per cent more generally used (95). McClary found 5 per cent dextrose to be superior to 2 per cent for corn roots (123). White examined the osmotic and nutritive roles of sucrose (217) and concluded that for his strain of tomato roots 2 per cent was approximately optimal and that it could not be replaced in any great part by sodium chloride. Tukey found that the concentrations required by excised peach embryos varied with age of embryo after fertilization (196). The effects of sugar concentrations on the growth and reversible vernalization of rye embryos was studied by Purvis (151) and on mitotic and elongation rates of wheat roots by Burström (29, 31). Related to the foregoing studies are those of Plantefol & Gautheret (149), Lachaux (110, 111), and Goris (80 to 83) on the interrelations between carbohydrate and respiration in excised tissues. Also, Gautheret showed that tissues of carrot can be adapted to utilize glycerol, although they die if placed on glycerol alone without previous adaptation in the presence of both dextrose and glycerol (74).

Vitamins.—Most excised plant tissues will make only a very limited "residual growth" (a term introduced by Carrel to designate increment at the expense of internal nutrition only) in a simple salt-carbohydrate solution. Corn roots appear to be an exception (123). The problem of providing additional accessory factors was in part solved by Kotte (101, 102) by using "Liebig's meat extract." Robbins employed peptone or autolyzed yeast (159). Subsequent studies have shown an extract of pasteurized brewer's yeast to be a more useful source of the required nutrient factors [White (201, 204); Fiedler (48); Gautheret (58)]. Analysis by White (206, 207, 208, 212), by Robbins & Bartley (162), Robbins & Schmidt (165 to 168), and by Bonner (7) and Bonner & Devirian (20) soon showed that this complex was completely replaceable by certain definite substances. Of these, thiamine is the most crucial [Robbins & Bartley (162); Bonner (7); White (208); Addicott (1)]. No tissues have been successfully grown without an external source of thiamine except in those cases, such as carrot tissue grown in the light [Nobécourt (137 to 140)] and corn roots [McClary (123)] in which it can be shown that thiamine is synthesized by the tissue itself. The concentrations required are very low, 0.1 p.p.m. furnishing optimal results while as little as 0.0001 p.p.m. (0.1 μ g per liter) will give detectable stimulation.

Besides thiamine, many plant tissues have one or more additional requirements. For tomato roots these can be met in any of three ways: (a) by use of 3 p.p.m. of glycine [White (212)]; (b) by use of a group of amino acids which, however, need not include glycine [White (207); Bonner & Addicott (16)]; or (c) by use of the vitamins pyridoxine [Robbins & Schmidt (166, 167, 168)] and niacin [Bonner (11); Bonner & Devirian (20); Whaley, Rabi-

deau & Moore (199)]. Bonner (15) and Day (40, 41) were unable to verify the utilization of glycine under their conditions of experiment. Yet in White's laboratory, tomato roots were grown for 4 years (1938 to 1942) in nutrients containing only glycine and thiamine in addition to salts and sucrose. They will grow equally well with thiamine and pyridoxine or with thiamine and a complex amino acid mixture, but not with thiamine alone. Apparently pyridoxine and perhaps niacin serve to mediate the synthesis of nitrates into amino acids and proteins, while in the absence of pyridoxine, amino nitrogen must be supplied as such [Bonner & Addicott (16)]. Robbins & Bartley (164) showed that some strains of tomato roots would grow if only the thiazole portion of the thiamine molecule was supplied. Bonner (8) studied the effects of a series of thiamine analogues on pea roots. Robbins (161) and Bonner & Devirian (20) also studied the effect of β -alanine on tomato roots but concluded that it was without importance.

Other tissues have more complex requirements. Pea roots require nicotinic acid in addition to thiamine [Addicott (2); Addicott & Bonner (3); Addicott & Devirian (4); Bonner (10)]. Bonner & Bonner showed that some, but not all, varieties of pea embryos require external supplies of ascorbic acid (18). In those cases where this vitamin is not required it can be shown that ascorbic acid is synthesized by the embryos. Many species of roots likewise synthesize riboflavin [Bonner (12)]. Other requirements have been studied in a series of papers by Bonner (6, 9, 13), Bonner & Addicott (16), Bonner & Axtman (17), Bonner & Bonner (18), Bonner & Buchman (19), and Bonner & Devirian (20). The requirements for carrot tissues were studied by Gautheret (59) and by Nobécourt (138). Gautheret's group, especially Morel, has studied the requirements of cambial tissues of a great many woody plants (127). Grape and many other vines require as accessory material only indoleacetic acid or its equivalent [see later (125, 126)]. Hawthorn requires pantothenic acid (129). Gautheret found willow to require both biotin and pantothenic acid (73), thus finally succeeding in growing a tissue with which he had worked unsuccessfully years before (51, 52, 53). Telle & Gautheret showed that *Hyocymus* root tissues also require pantothenic acid (191). Gioelli (78) and Cappelletti (36) studied the effects of follicular hormones on plant tissue growth. Bonner studied the production of riboflavin by roots of tomato, *Datura*, clover, alfalfa, and sunflower (12) and the inhibition of growth of tomato roots by the sulfa drugs which is reversible by *p*-aminobenzoic acid (13). De Ropp investigated the behavior of excised crown-gall tissue of sunflower with respect to the folic acid antagonists (181) and Slankis studied the role of extracts of *Boletus* as possible agents in the production of mycorrhizal nodules on pine roots (187). Vitamin requirements have been studied in cursory fashion for asparagus stem tips [Loo (117, 118)], dodder stem tips [Loo (119)] and for pine roots [Loo & Wang (120)]. Most of these experiments have been qualitative, without precise details being presented on the quantitative evaluation of the results.

Organic supplements other than vitamins.—While all the tissues extensively studied so far can be maintained with the salts, carbohydrates, and vitamins

discussed above, without additional supplements except for, in some cases, the auxins (see later) there are a number of other substances which, for one reason or another, have been investigated. Hildebrandt & Riker made a very extensive survey of possible carbon sources—sugars, organic acids, fats, etc. (94)—and Riker & Gutsche made a corresponding study of organic nitrogen sources, amino acids, purines, etc. (158). Two of their findings deserve special note. They found that certain amino acids, notably alanine and aspartic and glutamic acids give double maxima at about 0.1 and 128 mM with pronounced minima at about 1 mM (158). This appears to be possibly the result of a double function, the amino acids serving at low concentration as intermediates in the enzymatic processes of transamination and at higher concentrations as sources of organic nitrogen and carbon. No proof for this possibility is presented. Secondly, these authors found (158) that the best sources of nitrogen were nitrate [compare with Robbins & Schmidt (165)], urea, and alanine, with good but not optimal results from ammonium succinate, glycine [compare with White (212)], and DL-aspartic acid. It is suggested that the lability of amino groups in urea might make it an excellent source of these radicals for protein synthesis. Since birds also utilize urea, this opens up some interesting possibilities for studies on animal tissue nutrition.

A second, less clear cut, line of investigation involves the use of coconut milk as a source of growth factors. This stems from the work of van Overbeek, Conklin & Blakeslee (144, 145) on the cultivation of excised hybrid embryos of *Datura* which, in nature would have perished due to failure of development of the endosperm. These authors reasoned that coconut "milk," being itself a liquid endosperm, might take the place of the wanting nutrient, provided that protein specificities did not interfere with its utilization. This proved to be quite true. However, Blakeslee & Satina (21) subsequently found the same nutrient qualities in barley malt. Duhamet has also used coconut milk in the cultivation of artichoke tissues (46). Van Overbeek, Siu & Haagen-Smit (146) made some preliminary studies of the chemistry of the material.

A rather bizarre turn has been given to the subject by Caplin & Steward (35). These authors found that non-cambial tissues of carrot, in contrast to the cambial tissues used by Gautheret, Nobécourt, and others, could not be made to grow in any of the usual culture media, but that addition of coconut milk permitted a very rapid growth. This supplement is needed only for the initiation of growth, not for its maintenance. The effective material, which is also found in malt, in wheat and corn ovules at the milk stage, and in a number of other sources, is stable to prolonged boiling at neutral or slightly acid reaction and is freely dialyzable, hence must be nonprotein and of relatively small molecular weight. These authors found that another refractory tissue, that of the potato tuber, could be caused to grow if a proper balance was maintained between the coconut-milk factor and the powerful growth hormone, 2,4-D. The effects of 2,4-D on growth of carrot and endive tissues had also been studied by Gautheret (66, 67). The exact significance of these

findings is not yet clear. It should be emphasized that carrot tissue of cambial origin can and has been grown for years, at equal or nearly equal increment rates, without coconut milk or any other unknown organic supplement (Gautheret, Nobécourt, and others). This factor is thus certainly not a general requirement but functions in connection with some particular process at a particular phase of tissue proliferation and under special conditions. Somewhat different conditions entirely obviate its necessity. In this respect, Jacquot (99) has made an interesting observation. Tissues of many plants, particularly the coniferous trees, contain high concentrations of polyphenol-oxidases and tannins which react with the metallic ions of the nutrient and with surface products of cell breakdown, preventing normal growth. If fresh explants of such tissues are placed initially on a nutrient containing caffeine for about 48 hr., these oxidases react with the caffeine and are removed, after which cultures transferred to a caffeine-free nutrient will grow satisfactorily. Wetmore & Morel (personal communication, unpublished) arrived at the same result by substituting ascorbic acid for caffeine.

AUXINS

While the auxins or growth hormones are not, strictly speaking, to be considered nutrients and are even, according to Gautheret, to be looked upon as sub-lethal poisons (61), they represent such an important factor in the maintenance of growth of isolated plant tissues that they must obviously be considered here. Success in cultivating normal tissues of cambial origin (carrot tissue) was only obtained by the use of indoleacetic acid [Gautheret (56); Nobécourt (134, 136)]. The role of auxins in maintaining growth has since been studied for artichoke by Raoul & Gautheret (157), for grape (125, 126, 130), Virginia creeper (128), and hawthorn (129) by Morel, for willow by Gautheret (73), for lupine by Duhamet (45), and for a series of other plants by Gautheret (68, 70). Slankis has studied the role of indoleacetic acid in the branching of excised pine roots (189). Gardiner investigated its effect on plant embryos *in vitro* (50). Thielman & Pelece (193), Friedman & Francis (49), Geiger-Hüber & Burlet (76), and Burström (30) examined the effects of auxins on roots without obtaining evidence of their being required. In fact, most roots do not require an external source of auxin and many have been shown to produce auxins [pea, van Overbeek (142, 143); van Overbeek & Bonner (147)] as do other tissues [Kulescha (105); Kulescha & Gautheret (108)].

Certain cases exist in which tissues, once started on an auxin nutrient, subsequently become capable of growing without it. In this respect they acquire one of the qualities of tumor tissues. This process, first described by Gautheret (64), has been called "habituation"—to the absence of auxin. It has subsequently been studied by him (72, 75), by Morel (130), by Kulescha & Gautheret (107), by Camus & Gautheret (33, 34), and others.

The production of auxin from tryptophan, by plant tissues, was studied by Kulescha & Gautheret (109) and by Kulescha (106). The interrelations of auxin and colchicine were investigated by Duhamet (44) and Martin (121,

122). The differential responses of normal and tumor tissues to auxins have been studied *in extenso* by de Ropp (176, 177, 179, 180), Hildebrandt & Riker (93) [see also Hildebrandt, Riker & Dugger (97)], Gautheret (63), and Kulescha (104). Lachaux has investigated the effects of auxin on respiration *in vitro* (111, 113). De Ropp also found that crude penicillin would stimulate the growth of sunflower tissues, a response which he attributed to its content of auxin as an impurity (174). Hirth & Rybak (98) found a growth inhibitor in tissues of *Pelargonium*.

UNCLASSIFIED

There are a great many papers in which nutritional problems have played a secondary but important role. Thus, the studies on the cultivation of excised embryos in general have contributed to our knowledge of tissue nutrition. Among these are those on cherry by Tukey (195), *Zizania* by LaRue & Avery (116), grains by Brink, Cooper & Ausherman (23) and Merry (124), iris by Randolph (155) and Randolph & Cox (156), and on corn by Haagen-Smit, Siu & Wilson (86). Also, the work on the vernalization of embryos *in vitro* by LaRue (114, 115), Purvis (150 to 153), Gregory & Purvis (84), and Gregory & de Ropp (85), and on leaf growth by de Ropp (172, 173, 175) have broadened this knowledge. A particularly interesting example is that of Curtis (38) and Curtis & Nichol (39), who showed that the presence of barbiturates in the nutrient would cause orchid embryos to develop in bizarre, tumoroid forms.

In this category should also be mentioned the studies of Nitsch on the growth of whole excised fruits of tomato (132, 133). Experiments on the effects of temperature [White (205) and Slankis (188)], pH [Gautheret (65, 69, 71)], x-rays [King (100)], heterotic vigor [Whaley & Long (198)], and other factors should be mentioned [see, for example, Robbins & White (169, 170); Cova (37); Bonner (14); Gioelli (77); Dormer & Street (42); Gautheret (57, 60, 62); Kotte (101); Nobécourt and Köfler (141); Kulescha (103); Kulescha & Gautheret (107); and de Ropp (178)].

Respiration is closely associated with nutritional processes in general. The respiration of excised plant tissues was studied by Plantefol & Gautheret (148, 149) and subsequently by White (220), Rabideau & Whaley (154), Lachaux (110 to 113), Newcomb (131), and Henderson & Stauffer (92).

RÉSUMÉ

A glance at the more than two hundred references cited in this paper, most of which have appeared in the past ten years, shows how enormously this field of investigation has grown. When the author first reviewed the subject in 1931 (200), the papers of Kotte (101, 102) and Robbins (159, 160) represented the only ones which gave even a promise of success. The contrast between 1931 and 1951 is striking. It is a promising sign that these 200 odd papers have come from such widely scattered laboratories. Nevertheless, one of the disappointing features of the material covered in this review has been the dearth of data on the functions of the various substances studied. There is nothing comparable to the detailed analyses of nutrient functions

developed by the *Neurospora* school, although the materials themselves are as easily manipulated in many ways as are the molds. They do lack the possibilities of easy segregation of genetically tagged cell strains. On the other hand, the sudden appearance in cultures of nutritionally distinct regions, such as those reported for the Virginia creeper by Morel (130), strongly suggest somatic mutations which might be capable of study freed of the problems involved in genetic segregations. Street and his colleagues (190) and Burström have shown that a detailed analysis of nutritional problems such as that of sugar assimilation can be made by these means. Certainly the foundation has been laid, and much should be done in this direction. It is still a wide, interesting, and relatively unplumbed field which undoubtedly has an important future.

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FROST, DROUGHT, AND HEAT RESISTANCE

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Since the work on frost resistance has already been covered up to 1941 (1), this review will start from that date. For the sake of uniformity, the same date will be used (with few exceptions) as a starting point for drought and heat resistance as well, though these subjects were not covered in the above publication.

The main reason for dealing with all three kinds of resistance in one review is the large mass of evidence pointing to a relation between them (1, 2), i.e., the development of resistance to one factor usually involves an increase of resistance to one or both of the others. This point will be discussed in greater detail below. Such a relationship can be expected to apply only when resistance is used in the sense of withstanding or tolerating the frost, drought, and heat in the very tissues of the plant, but not in the sense of opposing these factors or preventing their penetration into the plant. Thus, it does not apply to the "drought resistance" of succulents, nor to the "frost resistance" of plants that supercool instead of freezing, nor to "heat resistance" due to an ability to maintain a lower temperature than that of the environment. This review will therefore be confined to resistance of the first kind mentioned above, with greatest emphasis on those papers that are concerned with the mechanism of resistance.

FROST RESISTANCE

Measurement and control of resistance.—During the past ten years, several reviews on frost resistance have appeared (3, 4, 5). There have also been numerous original investigations. The practical importance of the problem is attested to by the large number of workers who have found it worth while to install and employ freezing chambers. They have uniformly found good agreement between the degree of resistance determined in these chambers and field survival (6 to 11). Many workers (12, 13, 14) have followed the progress of hardening and dehardening in the field and the environmental factors controlling these processes. Factors affecting field survival have been investigated by Brierley & Landon (15, 16, 17), Shifton (18), and Rogers (19). Rogers & Modlibowska (20) were able to prevent frost injury to fruit blossoms by sprinkling with water, but this method was used only for moderate radiation frosts. The ice formed on the blossoms maintained their temperature 1 to 3°C. above that of the control dry flowers. Such methods, of course, do not increase resistance, but simply avoid or reduce the severity of the frost.

Rate of freezing and frost injury.—Apparently contrary to the well-established

lished relation between speed of freezing and frost injury (1, 4) is the statement by Mitchell (21) that rate of cooling (which may or may not have affected the rate of freezing) did not appreciably alter the resistance of guayule. The basis for this statement is an experiment in which plants were completely killed when the air temperature was gradually lowered from 63° to 24°F. over a six-hour period and the latter low temperature maintained for 5 hr. Earlier work by many investigators has shown that plants tender enough to be killed by such moderate temperatures are not affected by the rate of freezing.

The importance in the case of more resistant plants is attested to by Meader *et al.* (9) who state that a slow rate of cooling of peach fruit buds enabled them to withstand temperatures appreciably below what they survive under natural conditions. Scott & Cullinan (10) concluded that the rate of temperature fall was one of the most important factors in frost injury to peach buds. That conditions for rapid freezing occur in nature is attested to by Eggert's (22) measurements of cambium temperatures in peach and apple trees. On the south side of the tree the temperature frequently rose to 60° or 80°F., or even higher; and these temperatures were as much as 50° to 55°F. higher than on the north side.

Brierley (23) observed an interesting case of frost injury that may have been related to the rate of cooling. Due to a heavy blizzard in November 1940, the lower scaffold branches of a Haralson apple tree were bent down, split, and covered with snow. Some were dug from the snow in January to prevent further mechanical injury and in doing so were subjected to a rapid drop in temperature from 25° to 30°F. in the snow to 0° to -10° F. in the air. By the end of May, these branches were dead; those that were left covered by the snow were uninjured. The top of the tree that had been above the snow at all times was uninjured. As Brierley points out, the snow-covered branches may not have hardened as much as the exposed part of the tree.

In all investigations of the rate of freezing, it is desirable to determine the actual plant temperatures, using methods such as are described by Ullrich and Mäde (24), Eggert (22), or Rogers (19).

Metabolism at freezing temperatures.—Due to reports that the hardening process may continue at subfreezing temperatures (1), it is important to know whether or not metabolism continues at a measurable rate at such temperatures.

Walter (25) summarizes the investigations of Reitzenstein and Zeller on the assimilation and respiration of plants in winter at low temperatures. Older workers had reported carbon dioxide assimilation at temperatures even below -20°C., but the more recent workers have shown that it decreased with a drop in temperature until it was practically unmeasurable even before the zero point was reached. Walter describes a method that was worked out to settle this point. Very small quantities of carbon dioxide are measured by this method even outdoors on frosty days. Change in carbon

dioxide content of the air is detected by a change in color of cresol red in a solution in equilibrium with the air (i.e., by a change in pH).

Herbaceous winter annuals (wheat, barley, spinach) had no winter rest period. Their assimilation stopped at -2 to -3°C . and their respiration at -6 to -7°C . Spruce went into a winter rest and failed to assimilate at 0°C . Below -3°C ., it showed no respiration. *Prunus laurocerasus* had no winter rest and showed a negative metabolic balance due to cessation of assimilation at $+4.5^{\circ}\text{C}$.

Factors associated with frost resistance.—The physiologists' hope that the geneticist will be able to find out just how many factors are involved in frost resistance is still unfulfilled. Worzella (26) has confirmed the conclusion of previous genetical investigations that there is more than one factor, a conclusion with which no physiologist will quarrel.

The secondary nature of environmental factors is illustrated by some of the recent work on the effect of fertilizers on frost resistance. As was found by so many of the earlier investigators (1), Worzella & Cutler (6) observed the greatest injury to wheat seedlings grown on high levels of fertility. These more tender plants were large and succulent. Brown & Potter (27), on the other hand, failed to detect any loss of bearing surface on fertilized tung trees, whereas the unfertilized ones showed severe winter injury, some being killed outright. Yet this is not due to an inherent difference between woody and herbaceous plants. Thus, Kennard (28) noted an increase in winter injury to defoliated sour cherry trees as a result of late summer applications of nitrate of soda. Sudds & Marsh (29) observed a severe cracking of the bark of apple trunks accompanied by radial loosening. They ascribed this injury to a fall application of nitrate. It is highly probable that in all cases where injury was greater, carbohydrate content was lower. In the case of the tung trees, a seven-year period of no fertilization may quite conceivably have produced the same effect on carbohydrate content as the late summer and fall fertilization did in the other cases.

A simple relation to morphological factors is still being sought. Dillman (30) observed that hardy flax varieties have a branched spreading habit of growth when fall sown, the less hardy varieties an erect growth with few or no basal branches, just as when spring grown. Younger tissues were generally more resistant, as has usually been found by other workers (1). Gardner (31) observed winter killing of adult leaves and shoots of juniper though the juvenile branches were uninjured. The latter had no advantage of location that could account for the difference.

Photoperiod is not a factor in the frost resistance of winter wheat according to Kneen & Blish (8) since plants hardened adequately under either increasing or decreasing day lengths. Straib (11) investigated more than 100 wheat varieties and was unable to find any strict relation between frost resistance during winter and shooting time when spring sown. Many frost hardy varieties developed fertile shoots very readily when spring sown. Smith

(32), on the other hand, noticed that although no winter killing of normal sweet clover occurred during the winter of 1938 to 39, the July planting that was grown under a 16-hour day length in the fall was completely winter killed.

Using Gortner's cryoscopic method, Chandler (33) determined the bound water content in Minhardi wheat. Changes were associated with hardening, but these were accounted for quite satisfactorily by the quantitative and qualitative changes in solutes (e.g., sugars and amino acids) as a result of the physiological response to environment. Thus he supplies one more set of experimental results to the already long list (1) that fail to find any correlation between total colloiddally-bound water in plant sap and hardness. Grandfield (34) obtained a close correlation between bound water and hardness in alfalfa but he used Rosa's dilatometer method, which does not distinguish between colloiddally bound and osmotically bound water. Consequently, his results can easily be explained by the increased sugar content due to starch hydrolysis. It would seem by now unnecessary to investigate this point any further. If there is a role of bound water in hardness, it is essential to measure the quantity in the protoplasm, not in the cell or plant as a whole (1, 4). Chandler's results also point to the inability of the cryoscopic method to measure colloiddally-bound water, since osmotic effects cannot be accurately taken into account.

It is now a well-established fact that sugar concentration and osmotic pressure of sap frequently run parallel to frost hardness. Fresh evidence of the sugar relation to hardness in wheat varieties has been produced by Kneen & Blish (8). But as pointed out earlier (1, 4) there are many exceptions to this rule, and these two factors must be considered a secondary line of defense. Further evidence of this conclusion has been produced by Siminovitch & Briggs (35) and Pisek (36).

Siminovitch & Briggs found the usual seasonal parallelism between sugar content and hardness but showed that both reducing and nonreducing sugars decrease in spring more rapidly than rate of loss of hardness. They also increase again in June in the absence of any increase in hardness.

Pisek investigated the changes in leaves of three evergreen species: *Rhododendron ferrugineum*, *Prunus cembra*, and *Picea excelsa*. In *Rhododendron*, osmotic pressure and sugar content ran parallel to seasonal changes in hardness. This relation was much less pronounced in the conifers [as was previously shown by Levitt & Scarth (37)]. Thus, though conifer hardness varied from a killing temperature of $-5^{\circ}\text{C}.$ to about $-43^{\circ}\text{C}.$, the osmotic pressure varied only from 17 to 23 atm. *Rhododendron* hardness varied from a killing temperature of -5° to $-18^{\circ}\text{C}.$, and the osmotic pressure changed from 14 to 22 or 25 atm. Even in *Rhododendron*, however, the relation between these factors and hardness did not always hold. The plants could be artificially frost hardened during early summer by exposure to low temperatures. Though this increase in hardness was slight, the sugar content rose to equal the winter maximum. Similarly, marked dehardening

was induced during winter by exposure to high temperature, though the osmotic pressure and sugars decreased but slightly.

All the above results are of value in confirming the older concepts of frost hardiness. The only investigation along new lines is that of Siminovitch & Briggs (35) and Briggs & Siminovitch (38). Following up his earlier cellular work at McGill University under the direction of Scarth, Siminovitch approached the problem from a biochemical point of view and isolated the proteins from the bark of locust trees (*Robinia pseudacacea*). He found a marked increase in the soluble proteins from a minimum in summer to a maximum in winter, the latter attaining a value about four times the former.

Electrophoretic analysis (38) revealed at least five components in the soluble proteins, of which component A and especially B were present in higher quantities relative to the others during winter. The transitions, however, occurred not during the hardening and dehardening periods, but prior to hardening and subsequent to dehardening, respectively. The typical midsummer pattern (minimum for fractions A and B) occurred when the total soluble protein was at a minimum and when cambium activity was at a maximum. It is interesting to note that Siminovitch & Briggs were able to use the plasmolysis resistance method [previously described by Siminovitch & Levitt (39)] as a direct measure of frost resistance.

Due to language difficulties, this reviewer is, unfortunately, unable to consider the Russian work on frost, drought, and heat resistance. One paper, however, was presented by Henckel at the recent Botanic Congress. During autumn, according to him, plants accumulate a considerable amount of hydrophobic colloids, particularly fats and lipoids, which accumulate on the surface of the protoplasm and the vacuole and lead to dehydration of the protoplasm. At the same time, the plasmodesms are absorbed into the protoplasm, which loses contact with the walls of the cell. In such a state, the protoplasm swells but little in water and is not injured by ice crystals in the intercellular spaces (40).

The basis for the above conclusions, as far as this reviewer was able to judge, was as follows. The "accumulation of hydrophobic colloids, particularly fats and lipoids," was based on staining reactions observed under the microscope. Such observations have been repeatedly made by others but the later investigations have led to the conclusion that some of the so-called fats were really tannins and that the evidence for lipid accumulation is questionable (1).

The "absorption of plasmodesms" is based on observations of slightly plasmolyzed cells. Siminovitch & Levitt (39), however, showed that the cytoplasmic strands joining the plasmolyzed protoplast to the cell wall are invisible in slightly plasmolyzed hardy cells but eventually become easily visible when the same cells are further plasmolyzed by use of stronger solutions. Some of these cytoplasmic strands are seen to join the wall at the pits and therefore represent extended plasmodesms.

Finally, the statement that "the protoplasm loses its contact with the

walls of the cell" was substantiated by photographs of stained and apparently killed cells. One picture of unstained cells showed what looked like plasmolysis. But even these cells appeared abnormal to this reviewer, for the usual very dark refraction line normally seen in living plasmolyzed cells was not evident. Such observations are reminiscent of the "frost plasmolysis" in the older literature that was presumably due to the cell contraction in frozen tissues as a result of extracellular ice formation.

DROUGHT RESISTANCE

Measurement of drought resistance.—The practical significance of drought resistance has been the main reason for many of the investigations. The use of drought chambers for determining resistance is becoming as standard a practice as the use of cold chambers for determining frost resistance (41 to 46). As an example, Carroll (45) hardened pasture and turf grasses by exposure to atmospheric drought on three successive days for a period of 5 hr. daily in a draft oven at 35° to 37°C., and a relative humidity of 20 per cent. Resistance was tested by exposure to the same atmospheric drought for 12 to 24 hr. and determination of survival after three weeks. Up to 80 per cent of the unhardened and only 20 per cent of the hardened plants were injured as a result of 18 hr. exposure to the drought. Grass from a high nitrogen section suffered greater injury than from a low nitrogen section.

With the aid of their drought chamber, Kenway, Peto & Neatby (47) showed that drought resistance of wheat plants is lowest between 6:00 A.M. and 12:00 M. Laude (48) had previously obtained the same result.

Drought resistance versus desiccation resistance.—The method used to measure drought resistance depends on just what is meant by the term. This is illustrated by the series of papers by the Darmstadt group under the direction of Stocker [for a complete bibliography see (50)]. These workers define drought resistance as the ratio of the yield under dry conditions to the yield under optimal conditions of water supply (51). Though this is a valuable practical definition, it has no specific physiological significance, since the resistance may be due to such a variety of factors. Later, Stocker (49) recognizes two kinds of drought resistance: (a) protoplasmic: the ability to remain alive at a low water potential (i.e., vapor pressure); and (b) constitutional: the ability to maintain its protoplasm at a higher water potential than that of the atmosphere. Yet at no time do the Darmstadt workers apply any test to determine which of these they are dealing with. On the contrary, they sometimes ignore their own evidence. Thus, Stocker *et al.* (51) concluded that a drought resistant oat variety owed its resistance to a better water supply on account of a larger root system and higher osmotic pressure. This would be a case of "constitutional" drought resistance. Yet in an earlier publication (52), they used these same two varieties to determine the protoplasmic differences associated with what they apparently tacitly assumed was "protoplasmic" drought resistance.

Stocker also differentiates between "phenotypic" and "genotypic" drought resistance. The former involves changes in the plant when exposed to drought, the latter involves differences between drought resistant and drought sensitive species and races. According to his later paper (49), it is only the "phenotypic" drought resistance that he attempts to explain on a protoplasmic basis. The "genotypic" is due to a large number of different properties. Thus, grains and beets owe their respective resistance to very different factors, since the resistant variety of the former has a higher osmotic pressure, the resistant variety of the latter a lower osmotic pressure than the respective sensitive varieties.

The Vienna group, under the direction of Höfler, have followed Iljin's distinction between drought resistance and desiccation resistance (*austrocknungsfähigkeit*) though defining the latter in a different way (53). This is a departure from Maximov's earlier terminology which used xerophytism for what the German workers call drought resistance, and drought resistance for what they call desiccation resistance. In this review, only such desiccation resistance (or Maximov's drought resistance) will be dealt with.

The danger in assuming that varieties of "accepted drought resistance" are actually resistant to desiccation has been demonstrated by several workers. Cook (54) found that resistant strains of *Bromus inermis* consistently had a larger number of roots and in most cases significantly greater root depths than the nonresistant strains. The actual water supply to the cells may therefore have been better in the resistant strains. Platt & Darroch (43) obtained a coefficient of correlation of only 0.510 to 0.518 between yield and survival from artificial drought. Kenway, Peto & Neatby (47) were unable to find any clear relation between the drought resistance of wheat varieties, as determined by a drought machine, and yield under field conditions.

Milthorpe (55) failed to find any difference in desiccation injury between two varieties of wheat, though one was reputedly drought resistant, the other drought sensitive. The seedlings were dried over H_2SO_4 for 3 to 81 hr. after growing for 3 to 17 days from the time of soaking. Both varieties proved to be highly drought resistant. There were three distinct age periods associated with different degrees of resistance: (a) from the dormant embryo stage until the coleoptile was 3 to 4 mm. long, the seedlings were completely resistant; (b) until the emergence of the first leaf the plants were not permanently injured by loss of as much as 98 per cent of their total moisture, though elongating roots were killed; and (c) at later stages less water had to be lost in order for tissues to be killed, and the growth rate was permanently reduced by a small loss of water. These stages of drought resistance appear to be related to the proportions of meristematic and elongated cells. The former apparently are completely resistant.

Measurement of desiccation resistance.—Iljin's quantitative expression for desiccation resistance is the lowest relative humidity with which the

plant can come to equilibrium without suffering injury. Migsch (53) defines it as the water loss that a plant can withstand without injury as a percentage of the saturation water content. Höfler *et al.* (53) call this the critical saturation deficit which they determine as follows.

Leaves or shoots are removed from a plant, weighed, then allowed to dry slowly in the laboratory. After various time intervals they are again weighed and then allowed to reabsorb water. In this way they find the maximum water loss that can be withstood without preventing return of turgidity. Finally, they determine the dry weight. At the same time the saturation weight is obtained on parallel samples. The following equation is used:

$$\text{saturation deficit} = \frac{\text{saturation weight} - \text{fresh weight}}{\text{saturation weight} - \text{dry weight}} \times 100.$$

If the fresh weight in the above expression is the natural fresh weight, the equation yields the "natural saturation deficit." If it is the smallest fresh weight (determined as above) that still permits turgidity to be regained, then the expression gives the "critical saturation deficit." Finally, by use of the following ratio, they determined the actual degree of droughting that the plant is subjected to in nature:

$$\frac{\text{natural saturation deficit}}{\text{critical saturation deficit}} \times 100.$$

All of these values varied for different species, and since the natural saturation deficit and the critical saturation deficit did not necessarily run parallel for different plants, the ratio of the two was also different for different plants, e.g., plants that have suffered a greater water loss may be farther from their critical point than some that had lost less. Thus they showed clearly that different plants in the same region are droughted to different degrees. Even at the end of a drought period, the xerophytes may be suffering only a moderate deficit which is far from the critical point. Thus desiccation resistance is no measure of drought resistance, which results from the sum of many factors controlling water exchange. Yet they conclude that it is one of the most important components and very convenient for quantitative investigations.

In the cultivated plants tested, the critical saturation deficit varied from about 70 per cent for *Medicago sativa* to about 41 per cent for *Soja hispida*. The former, therefore, had the greatest desiccation resistance, the latter the least.

Since Iljin showed that death during desiccation may apparently be due to a mechanical tearing of the protoplasm, Höfler (56) concluded that there is no evidence that desiccation resistance of a cell is due to desiccation resistance of the protoplasm. Consequently, he felt it necessary to find out whether or not different kinds of protoplasm do differ in desiccation resistance. But he concluded that higher plants did not offer good material for

such tests, since Iljin showed that death of their cells on drying depends more on the nature of the cell wall and the size of the vacuole than on the inner properties of the protoplasm. In the liverworts, according to Höfler (57), these complications do not enter. Consequently, he investigated a large number of species of Jungermanniales. He placed the plants in chambers over H_2SO_4 of different controlled concentrations for 24 to 48 hr., then re-moistened them. Later (57), he took the precaution to permit reabsorption from the atmosphere in order to prevent mechanical injury. If the cells were alive after these treatments, the oil drops in the cells remained unaltered, if the cells were dead the oil drops disappeared or broke up. The different species showed markedly different thresholds of resistance, varying from fully resistant (survived over concentrated H_2SO_4) to fully sensitive (killed over 5 per cent H_2SO_4 , i.e., a relative humidity of 95.5 per cent). The resistance of a species was not constant, but the range of variation was small. Younger parts were more resistant than older ones, though the younger leaves frequently had the lower osmotic pressures.

Factors associated with drought resistance.—Maki *et al.* (58) attempted to increase the drought resistance of pine seedlings by inhibiting their growth with naphthaleneacetic acid. The growth of Jack pine seedlings was in this way reduced to 42 per cent of the controls and that of Loblolly pine to as little as 10 per cent of the controls. The seedlings were transplanted and left unwatered for 1 month and 17 days, respectively. In both cases the inhibited seedlings showed greater drought injury than the controls.

Biebl (59) concluded that boron increases the drought resistance of plants. He compared *Pisum sativum* and *Cucurbita pepo* plants grown with and without additions of boron. The plus boron plants showed the following characteristics of xerophytes: better developed root systems, higher moisture content of leaves, higher transpiration rates, and longer periods of stomatal opening, but no increase in succulence or in osmotic pressure. No direct observations of desiccation resistance were made. Furthermore, the above characteristics are not necessarily related to desiccation resistance.

Whiteside (60) found that the cells of wheat plants were smaller when grown with low moisture supply. The leaf size, distance apart of the stomata, and other such morphological characters were similarly affected. Lal & Malhotra (61) divided sugar cane varieties into three groups according to cell size. One variety, Rheora, is known to be drought resistant and belongs to the group with the smallest cell size. Therefore, they suggest that the lower the "cell index" the greater the drought resistance. Plausible as their conclusion is, the evidence they present is hardly adequate proof of it.

Many investigators have shown that the osmotic pressure of plants rises with increased drought (62). Using the plasmometric method, Schmidt found a 10 to 100 per cent increase in the case of droughted plants of *Lamium maculatum*. Whiteside (60) observed an increase in two wheat varieties from 16 atm. when well watered to 23 atm. when droughted. Bartel (63) found

that a soil moisture deficit led to an increase in osmotic pressure in four wheat varieties, the increase being in the order of the accepted differences in drought resistance.

Schmidt *et al.* (52) concluded that osmotic pressure is no criterion of drought resistance, since the relation between the osmotic pressure of tender and hardy varieties fluctuates markedly with external conditions. Furthermore, results varied with the species. Thus, in grains the resistant varieties had lower osmotic pressures under favorable moisture conditions, higher osmotic pressures under unfavorable moisture conditions. In the case of sugar beets the resistant variety always had a lower osmotic pressure than the tender one. These results are similar to what has been found in the case of frost resistance. However, until desiccation resistance is measured in these plants, it may be objected that perhaps the resistant beet variety owes its resistance to some factor such as a more extensive root system instead of to desiccation resistance.

Höfler *et al.* (53) found that drought resistant species in general have a high osmotic pressure. Yet less resistant ones may also have high osmotic pressures and resistant ones low osmotic pressures. Therefore, osmotic pressure cannot of itself be used as a criterion of drought resistance.

As in the case of frost resistance, earlier investigations led to the conclusion that bound water content was correlated with drought resistance (64). Several investigations soon followed, leading to conflicting results. Recently, Whitman (65) investigated the seasonal changes in bound water content of prairie grasses by means of the calorimetric method. Although the total water content decreased progressively and the percentage of total water bound increased, the bound water per gram dry matter failed to show any uniform change but fluctuated markedly from one test to another.

It seems likely that, as in the case of frost resistance, bound water can be uniformly related to drought resistance only if protoplasmic bound water is measured, rather than that of the plant or cell as a whole. On the basis of this hypothesis, Todd & Levitt (66) chose *Aspergillus niger* instead of a higher plant, because of the fact that the cells consist mostly of protoplasm. Consequently, any determinations of bound water on this plant are likely to apply primarily to the protoplasm. In higher plants, on the other hand, vacuole and cell wall make up a much larger part of the cell and the plant than does the protoplasm.

The fungus was grown in cultures containing varying amounts of dextrose besides the necessary mineral nutrients. In this way, media with osmotic pressures from 7 to 132 atm. were obtained. The bound water was determined simply by drying the washed and blotted mats at room temperature in a vacuum over a desiccant until equilibrium was reached, then drying in an oven at 80° or 110°C. The difference between the two weights was considered to be bound water and this value was of course higher when 110°C. was used than when 80°C. was used. In all cases, the bound water of the

mycelium rose with the osmotic pressure of the medium in which it was grown (from 1 to 2 per cent to about 30 per cent). The differences were large and the bound water in the mycelium grown at higher osmotic pressures was much higher than that of sugars. Consequently, it seems certain that only the proteins could be responsible for the changes in bound water. The isolated proteins are now being investigated from this point of view. The great difficulty is to obtain them in the undenatured state.

As in the case of frost resistance, investigators have recently turned to protoplasmic factors as possible causes of drought resistance. Levitt & Scarth (37) found an increase in permeability to urea of more than 100 per cent in cells of *Spartium* plants unwatered for 14 days. Whiteside (60) observed an increase to three times in the cases of two wheat varieties droughted for 12 days. During this time he showed that the desiccation resistance of the cells increased from a threshold humidity with osmotic pull of 45 to 60 atm. to one of about 100 atm.

The Darmstadt workers have obtained results that are not in agreement with the above. The permeability of droughted and moist grown *Lamium maculatum* plants was determined by Schmidt (62) using both the deplasmolysis and the plasmometric method. The droughted plants proved to be of the "glycerine type" (more permeable to glycerine than to urea), the moist grown plants of the "urea type" (more permeable to urea than to glycerine). But this difference was not constant since the droughted plants were converted to the "urea type" in the fall. The droughted plants were less permeable to urea, thiourea, methylurea, malonamid, and lactamid than were the moist-grown plants, but more permeable to ethylene glycol, glycerine, and erythrite.

Schmidt explains the reduction in amide permeability on the basis of the ultrafilter theory of permeability, i.e., as due to a decrease in size of the pores of the plasma membrane. The increase in alcohol permeability he explains by an increase in lipid permeability. On the basis of conclusions of other workers he suggests that the surface lipids are more basic in the droughted plants, more acid in the moist-grown plants.

The permeability results of Schmidt *et al.* (52) are confined to oats since wheat failed to give significant results and the beet cells were too small for experimentation. The resistant variety had a higher permeability to water, urea, and glycerine than the tender variety. Yet droughted plants showed a decrease in water and urea permeability and an increase in glycerine permeability. But not all the results agreed with this generalization. Thus potassium-deficient and nitrogen-deficient plants when droughted had a higher water permeability than when grown with adequate moisture.

Schmidt (62) determined the protoplasmic viscosity of *Lamium maculatum* plants in the droughted and moist-grown condition. Using the plasmolysis-time method he found that the droughted plants had two to three times the protoplasmic viscosity of the moist-grown. The centrifugal method

gave similar results (2 to 2.3 times). As he points out, the higher viscosity may be purely physical, as a result of the increase in osmotic pressure of the cell sap, causing a dehydration of the protoplasm. It is interesting to note that Levitt & Siminovitch (67) obtained similar results when comparing frost hardy and nonhardy cells, i.e., the lower consistency of the nonhardy cells was obtainable only when these were in equilibrium with a much lower dehydrating force than the hardy cells.

In the case of oats (52), the plants grown under conditions of drought always had more viscous protoplasm than those grown under conditions of adequate moisture. As in the case of *Lamium maculatum*, the difference is apparently due to the increased osmotic pressure. Thus, a 15 to 22 per cent increase in viscosity was obtained in the droughted plants, but it was necessary to use a plasmolyte 13 to 47 per cent higher in concentration. However, in the case of sugar beets this complication did not enter, i.e., resistant beets yielded higher viscosity values than tender beets, though the latter had the higher osmotic pressure. Five varieties of summer wheat showed the same relation between hardness and protoplasmic viscosity save that the most tender variety had the highest viscosity. But these varieties were not drought-hardened previous to testing, and their resistance was not determined. They were graded according to the field experience of the grower.

Theory of drought resistance.—On the basis of these results and of other results [Fischer *et al.*—see Schmidt *et al.* (52)] revealing a higher calcium and reduced potassium content in leaves of droughted plants (which they assume to be true of the protoplasm) Schmidt *et al.* propose the following theory.

Increased sugar content on droughting causes an increased osmotic pressure and a consequent increased protoplasmic viscosity (due to the strongly hydrated sugar molecules) as well as a reduced "pore permeability." An increase in the ratio of calcium:potassium causes a reduction in charge of the protein framework and of the colloids in the intermicellar fluid. There is consequently a reduced hydration and a freeing of lipoids. Due to the drought, the freed water is mostly removed. An increased viscosity and reduced pore permeability (to water and urea) results. The freeing of lipoids increases the lipid permeability to glycerine.

They admit that their explanation is opposed to that of Kessler & Ruhland. The latter workers observed an increased viscosity on frost hardening and concluded it was due to greater hydration and ion charge. But Schmidt *et al.* (52) argue that in the case of frost hardening there is no dehydration such as occurs during drought hardening.

Stocker's hypothesis of the fundamental importance of the calcium:potassium ratio as a cause of changes of protoplasmic viscosity and cell permeability is based solely on gross analyses of leaves, which reveal nothing about the ratio of the ions in the protoplasm. Furthermore, this theory cannot apply to frost resistance (and therefore it is almost certain to be inapplicable

to drought resistance—see below) since twigs can be removed from trees and dehardened completely in the laboratory without permitting any total loss or gain of any mineral elements (by simply keeping the twigs in a moist atmosphere). It seems certain that drought resistance can be lost in the same way. Pisek (36) actually found very little seasonal change in ion content of conifers, though a marked calcium and acid enrichment occurred in *Rhododendron* during one winter. Hardening and dehardening had no influence on the content of individual ions, and both conifers tested had very low quantities of calcium and magnesium.

Stocker's conclusion that hardening to drought causes a dehydration of the protoplasm is opposed by the results showing a marked increase in bound water of *Aspergillus niger* mycelium with increase in osmotic pressure of the medium.

Later, Stocker (49, 50) attempts to explain drought resistance on the basis of the molecular structure of protoplasm. He states that Iljin's conclusion that drought injury is mainly mechanical is upheld and extended by Kahl's shake investigations conducted under Stocker's supervision. A mechanical shaking of the plant produced the same physiologic effects as drought. After half an hour in the shake apparatus, protoplasmic viscosity was lowered and permeability to water, urea, and glycerine increased. Hydration capacity, transpiration, and respiration were increased while carbon dioxide assimilation and pH were lowered. (The methods used to measure these quantities are not described by Stocker, but it seems unlikely that hydration capacity and pH of the protoplasm were measured as implied below.)

Stocker explains these effects as due to a thixotropic gel→sol transformation. After shaking is stopped, the stimulation disappears and the previous equilibrium is reconstituted. In the same way, he concludes, desiccation causes mechanical tensions in the protoplasm, due to an unequal hydration capacity of different parts of the protein framework. Sooner or later this leads to a loosening and tearing of the network. These protoplasmic changes lead to the following drought reactions: (a) due to enlargement of the mesh, structural viscosity is lowered, pore permeability is increased, and with it transpiration; (b) by freeing of previously joined fibrils, formerly blocked polar and ionizable groups become effective and cause an increased hydration capacity and negative charge of the protoplasm; (c) freeing of enzymes causes an increased respiration and a hydrolytic cleavage; (d) due to a structural injury to the chloroplasts, assimilation is inhibited leading to a lowering of the pH; and (e) the lowering of the pH reduces the potential difference within the protoplasm, the fibrils reapproach each other, and are then able to reform the broken bonds thus reconstituting the original condition of the protoplasm. This is what he calls the restitution phase.

A rapid water removal, Stocker argues, must produce the above changes. But the drought stimulus is effective only during water removal. After

reaching a new, lower water equilibrium, the "restitution phase" is attained. Restitution leads to over-compensation of the drought reaction and the plant then enters the hardened condition. This occurs in "dry cultures" that are exposed to continuous strong atmospheric drought. The experimental results obtained by his co-workers applied to this restitution phase and therefore the results were quite different from those obtained by the shake apparatus, i.e., increased viscosity, lower permeability to water, and urea, increased permeability to glycerine.

Due to freeing of the blocked polar and ionizable groups, the imbibition capacity of the protoplasm is increased but cannot be satisfied because of the simultaneous water removal. Consequently, there is an increased uptake of divalent cations (calcium and magnesium) as shown by the reduced ratios of potassium:calcium and potassium:magnesium. Since the bivalent salts of proteins do not dissociate as much as the monovalent, a further decrease in the negative charge of the protoplasmic framework occurs, leading to the restitution phase. In the hardened state, transpiration is decreased due to reduced water permeability. There is also an improved metabolic balance due to inactivation of catabolic enzymes.

With regard to these newer aspects of Stocker's theory, the effects of shaking and the presumed similar effects of rapid water removal agree with the previously published work by Northen (68) with which Stocker is apparently unfamiliar. Northen showed that when species of *Mnium* and *Bryum* were dried for periods up to 50 min. over anhydrous CaSO_4 , thus producing "incipient drought," there was a decrease in structural viscosity which he ascribed to protein dissociation. He concluded that this assumed protein dissociation induced an increased protoplasmic swelling pressure, which he apparently believes is directly responsible for drought resistance. He does not propose the numerous opposite changes involved in Stocker's "restitution phase."

It is as well to recapitulate and evaluate the experimental evidence on which Stocker's earlier and later theories are based. These are (a) changes in protoplasmic viscosity, (b) changes in permeability, and (c) changes in ratio of calcium:potassium.

In the case of both oats and *Lamium maculatum*, the Darmstadt investigators found no evidence of any intrinsic change in protoplasmic viscosity associated with drought resistance but solely what may be accounted for by the dehydrating effect of the higher cell sap concentration. Since they admit that this increased cell sap concentration is only a secondary factor in drought resistance, we must conclude that the primary protoplasmic factor in the above plants is not revealed by viscosity measurements. Of course, they do not even know whether these drought resistant plants possess desiccation resistance. On the contrary, they showed that in the case of the two contrasting oat varieties, the drought resistant one owed its resist-

ance to a larger root system, higher osmotic pressure, etc. (51) and therefore might not have possessed superior desiccation resistance.

It should be pointed out that the important comparison of viscosities between hardy and sensitive cells is not when these are in the hydrated state but when they are in the dehydrated state. It is only when compared at the same degrees of dehydration that consistent differences between frost hardy and nonhardy (and the same must be true of drought hardy and nonhardy) protoplasm can be obtained (67), and these are in the opposite direction from those claimed by Stocker for normally hydrated resistant protoplasm. When the cells are highly hydrated, the hardy protoplasm may conceivably have the same, lower, or higher viscosity than nonhardy protoplasm, depending on the degree of hydration and on the balance between structural and true viscosity. In the case of dehydrated protoplasm only structural viscosity remains.

The second basis for Stocker's theory—the permeability difference—is equally unreliable. He concludes that drought hardening decreases permeability to water and urea, but that "genetic" drought resistance is associated with higher permeability to these substances. Since genetically controlled differences in protoplasmic drought resistance are evident only following a period of drought hardening, it is inconceivable that the two should be associated with opposite permeability differences. To add to the confusion, the Darmstadt workers conclude that the "genotypic" and "phenotypic" drought resistance are both associated with the same viscosity increase, yet the former involves high permeability to water and urea, the latter low permeability. Their own results do not uphold such conclusions, since potassium-deficient and nitrogen-deficient plants showed an increase in permeability when droughted. This is in agreement with the results obtained by Levitt & Scarth (37) and by Whiteside (60). Furthermore, Whiteside showed that the cells he used simultaneously increased in desiccation resistance. In view of the fact that the Darmstadt workers in no case determined desiccation resistance, the likeliest explanation of their series of opposite permeability relationships is that in some cases they were dealing with desiccation resistance, in other cases they were not.

In view of the lack of sound experimental evidence for Stocker's theory it seems unnecessary to consider the many inconsistencies in the theory itself point by point. On the basis of frost hardiness studies, this reviewer believes that a stronger case exists for a theory diametrically opposed to Stocker's, i.e., that hardiness is associated not with a tighter but with a looser binding of the protoplasmic fibrils, due to an increased hydration.

HEAT RESISTANCE

Measurement of heat resistance and its importance in nature.—Various methods have been used to measure heat resistance. Sapper (69) deter-

mined the maximum temperature (i.e., the highest temperature that failed to produce visible injury) by exposing the plants to saturated air for periods of one-half hour. For aquatic and shade plants, the maximum temperature was about 40°C., for xerophytes about 50°C. These differences in resistance between ecological types persisted, in general, when lower temperatures were used for longer periods. Lorenz (70) plunged sections of tissues into a water bath at known temperatures for known lengths of time, then by vital staining and plasmolysis determined whether or not the cells were alive. Heyne & Laude (71) exposed corn seedlings to a temperature of 130°F. and a relative humidity of 25 to 30 per cent for 5 hr. Their results indicated that testing for heat resistance can be relied upon for distinguishing genetic differences in drought resistance as determined by field survival. Only one inbred line differed widely in laboratory reaction and field test. But it should be pointed out that these tests may actually have measured desiccation injury, due to the very high vapor pressure deficit of the air. Sapper's precaution of using saturated air is to be recommended, if heat resistance alone is to be measured.

Julander (72) tested the heat resistance of range grasses by cutting stolons into 1½ inch lengths, leaving the root on them, transferring them in lots of eight to stoppered glass tubes, then immersing the tubes into a constant temperature bath at $48^{\circ} \pm 0.1^{\circ}\text{C.}$ for periods of 0, ½, 1, 2, 4, 8, and 16 hr. The stolons were then planted and recovery estimated after four weeks. Distinct differences in heat resistance were established, both between treatments and species. Soil measurements revealed that in overgrazed range the temperature rose to as high as 51.5°C., though this was not maintained for long. Yet the accompanying air temperatures did not exceed 36°C. Since under severe drought conditions air temperatures of 43°C. are not uncommon, soil temperatures must rise above these recorded values and heat injury is certain to occur. Thus, high soil temperatures may be a direct cause of the death of forage plants during drought, at least in the case of overgrazed range (see below).

Konis (73), on the other hand, concluded that there is no danger of direct heat damage to maqui plants under natural conditions in Palestine. He tested the still attached shoots of nine species in the field and found that the lethal temperatures were several degrees higher than the highest temperatures recorded in the field. Thus 4 to 5 hr. at 56°C. or 30 min. at 59°C. failed to damage *Rhamnus alaternum* and the highest leaf temperature recorded (by means of a thermocouple) in the field was 52.5°C. He measured heat resistance in the field by enclosing attached shoots in a blackened container. The temperature inside the container rose to as high as 60°C.

Similarly, Mueller & Weaver (74) were unable to detect any injury to prairie grasses subjected to hot winds of 135°F. when soil moisture was available. Even temperatures as high as 145°F. scarcely injured them. However, since actual leaf temperatures were not determined, these results are

not conclusive evidence of heat resistance, for the rapidly transpiring leaves may have had temperatures well below that of the atmosphere.

Carroll (75) raised both soil and air temperature (independently) to 40, 50, and 60°C. High soil temperature was more destructive than high air temperature.

Factors affecting heat resistance.—Sapper found that heat resistance was not increased by keeping the plants at high temperatures. On the other hand, those conditions that lead to adaptation to low temperatures (drying out and increase in osmotic pressure) at the same time raised heat resistance. Long starvation reduced the heat resistance but small differences in carbohydrate nutrition were without effect. When nutrient deficiency led to an increase in osmotic pressure, heat resistance was also increased. Excess mineral nutrients always reduced resistance.

Heat resistance was markedly influenced by the water content of the plant. Both gradual and rapid dehydration of protoplasm (by dry culture and wilting respectively) often produced a significant increase in heat resistance. But this dependence on water content held only when the same kind of protoplasm was observed under different conditions. When different species of plants were compared, those with the higher osmotic pressure, and therefore the lower water content, were not always more heat resistant. Heat resistance therefore depends, in the first place, on specific protoplasmic properties and is modified secondarily by water content.

These observations of Sapper's have been confirmed and extended by many other workers. Scheibmair (76) found that plasmolysis of moss cells by sucrose or salt solutions increased the heat resistance in three species and ascribes this to the dehydration. A fourth species had its heat resistance lowered by plasmolysis, supposedly due to mechanical injury not associated with heat injury.

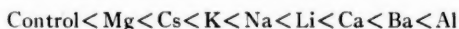
Heyne & Laude (71) found 10 to 14 day old corn seedlings more resistant than older ones. This was perhaps associated with exhaustion of the food material in the endosperm at about the 14th day. The heat resistance of seedlings kept in the dark for 12 to 18 hr. was increased significantly by an exposure to light for as short a period as 1 hr. As mentioned above, this same result has been claimed for drought resistance, which is what Heyne & Laude may have been measuring.

Julander (72) showed that plants grown under dry conditions required much longer heat treatments before being killed than did watered plants, e.g., 4 hr. produced complete killing in the case of watered bluestem, 16 hr. in the case of droughted bluestem. There was little difference between the species when they were in the unhardened condition, i.e., watered and clipped. Unhardened bluestem, slender wheat, Bermuda grass, and buffalo grass were killed by 2 to 4 hr. at 48°C. Blue grass, however, was more sensitive and a temperature of 45°C. was used for it. In the hardened condition there were definite differences. Bermuda grass and buffalo grass were

the most resistant and were not killed by the longest period (16 hr.) at 48°C., bluestem was intermediate and slender wheat, smooth brome, and Kentucky bluegrass were low in resistance. All species were susceptible to heat injury whenever the food reserves were low (e.g., as a result of clipping or grazing). The drought-hardened plants had about twice as high carbohydrate contents as the unhardened and very little starch. Sucrose accumulated while reducing sugars did not. The greatest accumulation was of colloidal carbohydrates, especially levulosans.

Theory of heat resistance.—Bogen (77) has proposed a theory on the basis of the effects of ions on heat resistance. He determined the time required for loss of color from anthocyanin-containing cells of *Rhoeo discolor* and *Gentiana cruciata*. Temperatures of 53° to 60°C. were used and the time for death at 60°C. varied from about 0 to 30 min. According to his observations, loss of color occurred before any protoplasmic change was visible. He therefore concludes that the first evidence of death is a pathologic increase in permeability.

Monovalent cations of equimolar concentration lowered heat resistance according to their position in the lyotropic series, i.e., $\text{Li} > \text{Na} > \text{K} > \text{Rb} > \text{Cs}$. Monovalent anions lowered heat resistance according to their order of adsorption, i.e., $\text{SCN} > \text{NO}_3 > \text{Br} > \text{Cl} > \text{C}_2\text{O}_4 > \text{SO}_4$. Multivalent cations did not follow the lyotropic series and he explains their behavior as due to the interference of the oppositely acting adsorption effect, i.e., a discharging of the colloid. Thus, when both monovalent and divalent cations are grouped together, the following is the order of effectiveness:



When he combined the anion and cation that were both least effective in their respective series (i.e., MgSO_4) he actually obtained an increased heat resistance. This was the only salt tested that produced an increase.

The change in heat resistance was proportional to salt concentration in an exponential manner, i.e., when the concentrations were plotted in a geometric series, a straight line relation was obtained with killing time. Mixtures failed to produce any antagonistic effect. Thus, $\text{KCl} + \text{CaCl}_2$ produced an additive effect, $\text{LiCl} + \text{MgSO}_4$ purely a MgSO_4 effect.

The above results were based on tests in which the sections were plunged directly in the solution at the high temperature. Bogen also determined the effect of pretreatments on heat resistance. Keeping the sections in water before exposure to high temperature reduced heat resistance, more rapidly in distilled water than in tap water, though the end effect (after prolonged pretreatment) was the same in both cases. Pretreatment with single salt solutions had the same effect as direct exposure to those solutions at high temperature. Hypotonic sugar solutions had no effect on heat resistance. Slightly hypertonic solutions increased resistance, strongly hypertonic reduced it. If the hypertonic sugar solution was replaced by tap water at 60°C.,

death was almost instantaneous. This rapid deplasmolysis was the most effective factor in reducing heat resistance.

Bogen attempts to explain all these results on the basis of modern knowledge of proteins, particularly the folding of proteins and the structural bonds between them. He assumes that heat resistance is determined by the stability of the molecular form and that any molecular deformation or tearing of molecular bonds can lead to a structural disturbance and finally to death of the cell. Thus he concludes that heat resistance is not due simply to the degree of hydration, but to the original order of charges and the hydration centers on individual molecules. Any disturbance of this order may lead to death, since the molecule is thereby deformed and the system of molecular bonds exposed to tensions. In this process, the total water content may be increased, decreased, or unaltered. This, he thinks, may explain the many contradictory results on the influence of hydration on heat resistance. Where heat resistance and hydration do run parallel, it is because they are similarly affected by many factors.

The effects of the monovalent cations, according to Bogen, can be explained only by adsorption which occurs without discharging the colloidal particle. As a result, the imbibition of the protein is increased, the molecular bonds are loosened, and heat resistance is lowered. The same is true for the monovalent anions since they are effective in the order of their adsorbability. In both cases, however, Bogen states that it is not the hydration of the colloid as a whole that is involved but of individual molecules, and even then not equally over the whole molecular surface. Nor is it likely to involve a simple enlargement of the hydration shell about previously existing hydration centers. More likely, new dipole moments are induced under the influence of the ion and new hydration centers are created. Thus, both the charge distribution and the arrangement of the hydration centers are altered. The increased heat resistance produced by MgSO_4 does not involve a change in hydration but is associated with strengthening of the structure. It may bind two protein molecules together and hold them in their original orientation, thereby stabilizing the system of molecular bonds. The effect of pretreatment with water he explains as due to the washing out of ions.

Bogen's theory does not agree with the results obtained for frost and drought resistance (see below). On the other hand, his own results do agree with the concept that increased hydration of the protoplasm causes increased heat injury, due perhaps to a more ready unfolding (i.e., denaturation) of the micelles in the presence of ample water. His series of anions reduce resistance in the order of their effects on swelling. It has long been known that salts of the alkali metals gradually penetrate the plasma membrane and cause the cytoplasmic layer to absorb vast amounts of water. This is shown by the great increase in thickness and the clear, highly fluid nature of the cytoplasm (80). The order of effectiveness of these alkali ions on protoplasmic hydration is the same as the order in which they reduce heat

resistance. Salts of calcium and magnesium have the opposite effect on hydration. This would explain the beneficial action of the magnesium (obtained by Bogen) but not the harmful effect of the calcium salts. However, Scheibmair (76) found that though potassium chloride reduced heat resistance, CaCl_2 increased it.

Another factor that may enter into Bogen's results is permeability. In order to produce effects on the protoplasmic framework, the ions must first penetrate. Thus, the protective effect of MgSO_4 may be due to the fact that it does not penetrate appreciably and therefore dehydrates the protoplasm osmotically. A loss of more highly hydrating ions from the protoplasm might also be involved (and is, in fact, much more likely to occur when external ions are present for exchange than in distilled water, as suggested by Bogen). Another factor that cannot be ignored is the toxic effect of the unbalanced ions, which might be far more pronounced at the higher temperatures. It may be significant that the effects of the alkali metals on heat resistance are in the opposite order of their penetration rates. It is therefore possible that the reduction of heat resistance is largely due to action of the external ions on the plasma membrane.

RELATIONS BETWEEN FROST, DROUGHT, AND HEAT RESISTANCE

Some of the results described above [Heyne & Laude (71), Julander (72)] indicate the possibility that what is usually considered drought resistance may sometimes be heat resistance. One complicating factor in deciding such an issue is the fact that drought hardening actually increases heat resistance (see above). Since many earlier investigations (1) also showed that drought hardening increases frost resistance, this has led to comparisons of all three.

The older work has been confirmed and extended by the McGill group. Thus Siminovitch (78) determined the desiccation resistance of frost hardy and unhardy cells by exposing sections to relative humidities of 50 to 100 per cent. At the same time, other sections were exposed to low temperatures to determine their frost resistance. Resistance to frost and desiccation injury always ran parallel. Whiteside (60) hardened wheat plants of different varieties both by soil drought and by low temperature exposure. In both cases the varieties were arranged similarly with regard to desiccation resistance. Scarth (2) has thoroughly discussed the parallelisms between drought and frost resistance, pointing out that both are cases of dehydration resistance.

On the basis of earlier observations by Nelson (unpublished) that certain orange peel cells are more heat resistant than others, Levitt & Nelson (79) tested the resistance of these different types of cells to heat, frost, drought, and plasmolysis injury. The three groups of cells—(a) epidermal and sub-epidermal, (b) gland, and (c) cortical cells—fell into the same order of resistance to the four sources of injury. The gland cells were the most resistant, yet they usually had a lower cell sap concentration and were always larger than the other cells. Thus the protoplasmic factor (tested by resistance to

plasmolysis injury) is more important than small cell size and high osmotic pressure which are frequently correlated with frost, drought, and heat resistance.

Carroll (45) investigated the effects of high and low nitrogen supply on drought, heat, and frost resistance of turf grasses. Fifteen species from six genera were used. Soil drought was obtained by drying to soil moisture contents of 5 per cent and 3 per cent (2.7 per cent and 4.7 per cent below the wilting percentage) over a period of 7 to 10 days. The soil was then watered to 70 per cent of its water holding capacity and survival noted after three weeks. Frost resistance was determined by exposure of plants (hardened for one week at 0°C.) to air temperatures of -25°C. until the soil temperature had reached -5, -10, -15, and -20°C. respectively, in different tests. The plants were then transferred to the greenhouse and survival noted after three weeks. The method of measuring heat resistance is mentioned above. The high nitrogen plants proved to be less resistant to drought, frost, and heat injury. Similarly, both bound water (determined calorimetrically) and sugar accumulation were lower in the high nitrogen series.

But the correlation between these factors and resistance was not always obtained. Thus it was not possible to find any relationship between either bound water or sugar content and the relative hardiness of the different species of grasses. Similarly, for some species the order of resistance to drought was the opposite of the order of resistance to heat. But these exceptions were in the minority.

Carroll attempted to show a further relationship between drought and frost resistance by hardening two lots of grasses at 0°C. for one week, one with optimum soil moisture, the other with the soil moisture slightly above the wilting percentage. When tested in the freezing chamber, the dry series showed no significant improvement in frost hardiness. Such an experiment, however, fails to yield information on this point. As Carroll points out, the temperature drop to -10°C. took 3½ hours for the high moisture series and 1½ hours for the low moisture series, and therefore the greater rate of freezing in the latter may explain the results. If the two series had been brought to the same moisture content just before freezing, results might have been different. However, it is possible that though the low moisture may of itself induce frost hardiness, it may perhaps fail to produce any additional hardening when superimposed on the low temperature treatment.

Comparison of theories of frost, drought, and heat resistance.—It is now clear that frost, drought (i.e., desiccation), and heat resistance are all basically similar, and that any resistance to one of these factors carries with it a resistance to the others. Consequently, a theory proposed to explain one of them must apply to all. Since three theories have been proposed in the past decade, one for each kind of resistance, it is necessary to compare them with each other.

Both Stocker and Bogen base their respective theories of drought and

heat resistance on the assumption that resistance is associated with a strengthening of the bonds attaching the protein fibrils to each other in the protoplasm. This would lead to the conclusion that resistant protoplasm is more rigid than nonresistant protoplasm, whereas the opposite is the case (39). The increase in soluble proteins on hardening (35) also disproves their concept.

The experimental basis of Stocker's theory was shown above to be non-existent, i.e., he has no consistent evidence of either the viscosity changes or the permeability changes that he lays claim to, and the calcium:potassium ratio has been shown not to behave as his theory predicts. Furthermore, there is no assurance that his experiments deal with desiccation resistance.

Scarath's (4) and Kessler & Ruhland's (81) theory of frost resistance and Stocker's (49, 50) theory of drought resistance are mutually incompatible. The former is based on a concept of increased, the latter of decreased protoplasmic hydration. The large body of evidence on which the former is based—increased permeability to polar substances, less pronounced increase in protoplasmic consistency as a result of dehydration, increased thickness of protoplasm layer, etc.—has been fully described in previous reviews (1, 4). The more recent evidence of an increase in soluble proteins during frost hardening and of an increase in bound water as a result of "osmotic hardening" are in full agreement with the above.

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EXPERIMENTAL MORPHOGENESIS IN VASCULAR PLANTS¹

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INTRODUCTION

This paper has been written in the hope that it may provoke the interest of physiologists, biochemists, and geneticists in the manifold problems of morphogenesis; for, as this review will show, a sustained cooperative effort is essential if progress is to be assured in this complex and challenging field.

A vascular plant presents a striking contrast in its development to that of a vertebrate animal. The latter undergoes gastrulation and in consequence envelopes its digestive and absorbing system, thereby forming a compact adult animal which must ingest elaborated food materials as an energy substrate. The former grows apically and continuously, extending itself at both its absorbing root tips and its leaf-bearing stem tips. The resulting leaves and associated green parts become the energy-providing organs. Thus the absorbing system is being continuously and further separated from the foliar or photosynthetic system. In the distal region of both shoot and root, an embryonic region, the apical meristem, exists. Under favorable conditions, mitotic activity is pronounced in these meristems. Proximal to each apical meristem, gradual and progressive histological changes become evident as development proceeds. Only once in its development does the vertebrate animal go through its embryological changes; but in the plant, by contrast, growth and developmental change are sustained throughout the vegetative life of each stem and root tip exemplifying what Bower (1, pp. 14-15) has called their "continued embryology."

Morphogenesis—the inception of form and structure—inevitably involves an inquiry into the continuing production of new cells and their subsequent growth and differentiation into the functioning tissues of the integrated and harmoniously formed adult organism. In short, it connotes organized growth. Thus, organogenesis and histogenesis are both included in the term. In all stages of development, gene-controlled biochemical and physiological processes are recognized as fundamentally important. Yet the impact of external environment on genic expression in form and structure must obviously be reckoned with in any consideration of morphogenetic

¹ Because of the limitations of space, the authors have confined this review to experimental morphogenetic studies on the apical meristems of the shoot and to the literature of the period between 1935 and 1950 primarily.

problems, whether or not the effects of the collective or individual factors comprising it are understood. The sequence of events in active apical regions has a very special importance in studies of morphogenesis in vascular plants.

At this point, we believe it necessary to define the term differentiation as it will be employed in this article. The basic or initial materials in growing plants are usually the densely protoplasmic, embryonic or meristematic cells. Differentiation is considered to cover any recognizable morphological or physiological change in a meristematic cell or cell aggregate. It has generally been accepted by the morphologist and anatomist that differentiation refers to visible change. Yet all biologists recognize that invisible changes must precede those which are visible. Nor, since they are concerned with plants, do the authors limit the concept to irreversible change only. Reversible changes, if and when they take place, are considered as instances of dedifferentiation. In its general connotation and in its implication in this article, differentiation is therefore employed essentially in the sense that Weiss (2, pp. 93-94) uses modulation.

Over the last hundred years, many investigators have directed their efforts to a descriptive interpretation of developmental changes at the apices of shoots and roots. These efforts have been especially concentrated in the past three decades. Modern techniques have enabled workers to describe and illustrate faithfully the apical meristems in species drawn from widely separated groups of vascular plants. During recent years also, considerable attention has been given to the correlated growth and development of the cells produced by the apical meristem into the integrated visible structures of the mature plant. There has, however, been scarcely any inquiry into the biochemical and physiological processes which must underlie and precede the visible morphological expression.

To a small number of contemporary workers it has seemed that though descriptive efforts constitute a necessary preliminary phase and though they reveal what and where some of the problems of morphogenesis are, they must be followed by experiments in which hypotheses relating to morphogenetic processes and relationships may be subjected to crucial tests. In these experimental studies, meristematic regions of the apex have been subjected to surgical treatments and to culture *in vitro*. The morphological or histological changes observed in the experimental materials are the manifestations of growth; hence, the student of morphogenesis must extend his investigations to a consideration of the relevant physiological processes. In short, there is an outstanding need for studies of the physiology and biochemistry of embryonic or meristematic regions of plants.

Had it been practical, the student of morphogenesis in plants might well have followed the example of his zoological colleagues and studied experimentally the morphology and physiology of developing zygotes. In practice, this has offered so many difficulties that the main body of information on morphogenesis in the vascular plants has been obtained from shoot and bud apices. Accordingly, in this article, reference will chiefly be made to these

embryonic regions. In passing it may be noted that recent and current investigations on plant embryo culture and embryogeny *in vitro* should add materially to our knowledge of morphogenetic processes. [See works of Bonner & Axtman (3), LaRue (4), LaRue & Avery (5), Merry (6), van Overbeek (7), van Overbeek, Conklin & Blakeslee (8), van Overbeek, Siu & Haagen-Smit (9), and Tukey (10, 11, 12).]

Apical meristems.—In the past 25 years our knowledge of the apical meristems of shoots has been greatly extended. At the present time, it can be said with some assurance that the foundations have been laid for a comparative study of the organization in the shoot apices of the vascular plants. Certain definite ideas have now become clear, of which perhaps the foremost is the recognition that apical meristems have no common histological pattern. They do not necessarily consist of an aggregation of small uniform unvacuolated cells as many botanical textbooks suggest. Nor in the comparative survey does any one type stand out sharply as the basic or primitive type from which the others may have been derived. The following summary of types of apical meristems is intended to carry no suggestion of evolutionary or other sequence; it merely lists the variations in histological constitution: (a) a single, large, superficial, initial cell; (b) two to several large but less conspicuous apical or initial cells; (c) a group of inconspicuous, superficial initial cells; (d) a weakly-zoned or layered pattern; (e) a zoned or layered organization with the number of layers varying seasonally; (f) a relatively stabilized layered construction; and (g) a radiating construction from a central, sub-surface group of "mother cells". [See Foster (13), Reeve (14), Philipson (15,16), Majumdar (17) for reviews and bibliography.]

Notwithstanding this variation in histological pattern, all apical meristems have certain features and morphogenetic activities in common. They all give rise to vasculated axes bearing regularly arranged leaf primordia and buds and they control the development of the buds. The evidence suggests that each apical meristem is a source of an over-all basipetal auxin gradient in the axis. All apical meristems, moreover, have an important action in the inception of the vascular system which is always initiated in close proximity to them. It is surely an amazing fact that such important actions are common to apical meristems possessing no histological pattern in common. Community of morphogenetic effect, therefore, is not based on histological uniformity. One may well ponder the fact that, notwithstanding much investigation since Wolff (18) first recognized the shoot apex, botanists have yet to provide an adequate account of the factors which determine the organization and morphogenetic activity of that characteristic, all-important, and perennially embryonic region, the apical meristem.

RESULTS OF EXPERIMENTAL TECHNIQUES

Surgical treatments.—In all groups of vascular plants, whether the stem be upright or procumbent, it terminates in an apical meristem; and, as we have seen, irrespective of the type of meristem, leaf primordia originate on

its flanks, whether one, two, or more at a time, in a regular sequence, thereby providing a characteristic phyllotactic pattern. The primordia arise on the meristem as small elevations, the youngest leaf (or pair or whorl) closest to the summit (or tip) of the shoot, and older leaves in order of age progressively further away and on different radii. In all cases, the central, distal area of the apical meristem is unoccupied by leaf primordia; it is this ultimate, embryonic portion of the apical region which may be held to constitute the apical meristem proper, though, in fact, it is exceedingly difficult to find a definition of it which can be applied with equal precision to the several diversely constituted apices.

As the foliar primordia make their appearance and gradually take form, so does the vascular system of the leaf become recognizable. In favorable materials it can be seen that the inception of the vascular strand is essentially contemporaneous with the first appearance of the leaf primordium. Moreover, it is quickly continuous with the adjacent incipient vascular tissue of the stem. At this early stage, also, bud primordia, identifiable as areas of superficial meristematic cells, can often be recognized in or near the upper axils of the leaf primordia in both ferns and angiosperms [Wardlaw (19, 20), Garrison (21, 22)].

Wardlaw (23) has shown for *Dryopteris aristata* that if the apical cell is punctured with a fine needle, thereby stopping growth of the apex, leaf primordia may continue to develop in normal sequence until all the apical meristem has been used up. The entire meristem is interpreted therefore as potentially capable of producing leaf primordia, though this does not take place in a normal, growing apex. As will be pointed out in more detail later, Wardlaw suggests that the evidence indicates an inhibitory zone against leaf formation for some distance around the apical cell group; only near the edges of this zone do leaves ordinarily arise.

In his early studies of shoot apices by surgical techniques, Wardlaw (19, 20, 24) found that decapitation of the rhizomes of *Matteuccia struthiopteris*, *Onoclea sensibilis*, and species of *Dryopteris*, resulted in the appearance of buds. As already indicated, these originated from areas of primordial cells, initially part of an apical meristem, which had retained their distinctive appearance and meristematic activity, but had remained in a morphogenetically inactive condition until the apical meristem had been removed. These "detached meristems," as Wardlaw terms them (19, p. 180), could give rise to single buds or to a linear series of buds. When a tangential cut was made from above downward in the rhizome close to the surface (24), so that the detached meristem was isolated from the apex and from the cortical tissue, not only did a plantling originate from the semi-isolated flap, but the underlying cortical tissue also gave rise to a bud. It is noteworthy that both types of these buds developed vascular tissue.

One observes with interest that even the puncturing of the apical cell (as a result of which some injury probably occurs to neighboring cells) is

enough to remove the inhibition of buds, for very shortly thereafter buds begin to appear in the expected positions.

The appearance of buds following decapitation of the main apex is well known in many species of plants. But the similar histogenic origin and development of buds after decapitation of the main apex in plants in which buds are normally rare calls for comment. It has, moreover, been found that the growth of buds on decapitated stems of a fern can be inhibited by an application of auxin to the cut surface of the stem. This phenomenon is well recognized in angiosperms [Thimann & Skoog (25, 26)].

In a second series of investigations, Wardlaw (27, 28) has explored the effect of destruction of leaf primordia on the differentiation of the vascular system. The experimental technique consisted of taking an apical piece, a few inches long, of the rhizome of a fern, *Dryopteris aristata* or *D. filix-mas*, exposing the apical meristem, and removing all the leaves and leaf primordia, including the very youngest of them. All later appearing leaf primordia were then destroyed by puncturing them with a fine needle or by other equally effective techniques as soon as they appeared. After several weeks, when the rhizome was fixed, embedded, and sectioned, it was seen that the characteristic, pith-containing vascular cylinder or dictyostele was no longer broken by parenchymatous leaf gaps; instead there was a continuous, uninterrupted solenostelic vascular system. When, after a period of time, the puncturing of leaf primordia was discontinued, a normal dictyostele with leaf gaps was found in the region of new growth. These studies led Wardlaw to infer that the stele in the fern is truly a cauline or axial structure and not merely comprised of a collection of decurrent foliar vascular strands as has often been suggested, and that the foliar gaps are directly related to the considerable tangential growth of the leaf-bases. Subsequently, this experiment was repeated with other species of ferns [*Osmunda*, *Todea*, and *Angiopteris* (29, 30)], and also with the angiosperm, *Primula polyantha* (31) with closely comparable results. Corresponding findings have been obtained by Wetmore and his students (unpublished as yet) on still other species of ferns.

In a third set of experiments, Wardlaw (32) attempted to determine the effect of isolating the apical meristem proper by three or four vertical incisions. In this way, the isolated apical meristem was not in contact laterally with the adjacent organs or tissues; it was, however, continuous with the pith tissue upon which it was seated.

In many such experiments, the isolated meristem continued to grow as an elevated plug of tissue, in fact as a short axis. Moreover, this axis extended itself at approximately its previous rate. Within a few weeks, new leaf primordia were being formed in the former phyllotactic sequence, both the new axis and its lateral members becoming vascularized, despite the fact that the new stele, at least for the duration of the experiment, did not become connected with the vascular system of the parent shoot.

Some interesting inferences can be drawn from the data of these investi-

gations. It is evident that the growing apex is not necessarily nourished by way of the incipient vascular tissue. On the contrary, it can receive its water, mineral salts, and whatever else it requires through the pith, for by this tissue only was the new axis connected with the parent shoot. Wardlaw also points out that here is clear evidence that the inception of vascular tissue in the new growth cannot be directly due to the presence of vascular tissue below or to substances moving upward into the apex through such vascular tissue. Rather, the evidence points to the importance of the apex as an organizing center, determining the differentiation of the tissue beneath it. He concludes (32, p. 372) that in the pteridophytes the inception or initial differentiation of vascular tissue is related to the downward diffusion of an unspecified substance or substances from the actively growing apical meristem.

The data of these experiments also contribute to our knowledge of the factors which determine the outline of the stele as seen in cross-sections of the shoot. Thus, when four cuts were used to isolate the apex, the plug had a rectangular transverse outline and a stele of rectangular outline was present; but when three cuts were employed, the plug was triangular and so also was its stele. Evidence is accumulating which indicates the importance of mechanical factors in morphogenesis (32, p. 372). In the materials under consideration, since the cortical and pith tissues of the plug grow more rapidly than the small-celled incipient vascular tissue, it is a natural consequence that the stele should have a shape corresponding to the external shape; for the developing vascular tissue will tend to be compressed between the expanding pith and the peripheral cortex with its definite shape.

A further conclusion from these investigations is that, in the fern studied, while the apical meristem determines the organization below it, including the initial tissue pattern, that pattern may be further and very considerably modified in the sub-apical region.

Wardlaw (33) called attention to the fact that the distance of the incisions which isolate a portion of the apical meristem from the apical cell in *Dryopteris* has much to do with the size of the solenostele in the developing plug of tissue. Thus, a plug of small diameter will have a solenostele of small diameter; and when the incisions were made very close to the apical cell, a protostele resulted. As important as this observation is, the subsequent fate of this small shoot is even more so: "the small shoot later became attenuated, the apical region became parenchymatous, and the vascular system faded out" (33, p. 443). It seems that the apical meristem can become too small to continue control of the initiation of the vascular system behind it. Many studies of the orderly development of the vascular tissues in isolated shoots have provided support for Wardlaw's (33) hypothesis that, in *Dryopteris aristata* at least, the initial differentiation of the vascular system is due to the downward movement of a substance or substances from an actively growing apical meristem. This, in more general terms, implies that the inception of vascular tissue is determined by the active apical meristem. Certainly in such instances it is difficult to conceive of an independent and

new vascular system being incited to develop in an isolated plug of tissue, with orderly differentiation of xylem and phloem, by "stimulating substances proceeding from the older, preformed vascular tissue" (33, p. 444). For the water and the nutritive and formative substances which reach the young new shoot from that part of the plant which is below the original incisions can do so only through a core of pith tissue.

The studies described above were originally made on a fern, *Dryopteris aristata*, a plant with a distinctive apical meristem and a single apical initial. It became of immediate interest to students of morphogenesis to know if, by using the same experimental technique, comparable results would be obtained with an angiosperm possessing a histologically different apical meristem, that is, one with a layered tunica and no recognizable apical initials. Ball (34) carried out such an experiment on the apical region of *Lupinus albus* and Wardlaw extended his previous studies to include the apex of *Primula polyantha* (31). Both workers found that the isolated portion of the apical meristem developed as did that in *Dryopteris aristata* giving a short axis bearing leaves in essentially normal phyllotactic fashion. Moreover, in both sets of experiments, the vascular tissue in this axis differentiated basipetally through the pith. It is to be noted especially that, in these two cases, the vascular tissue of the new growth ultimately established connection with the conducting tissue of the original axis below the incisions. It becomes evident, therefore, that apical histological organization per se is not important in determining the pattern of the vasculated leafy shoot to which it gives rise. For in these two different groups of plants, ferns and angiosperms, with two histologically very different types of apical meristem, similar surgical experimentation produced closely comparable results. Moreover, this new and independent vascular tissue, in the two angiosperms, has been found to become continuous with that of the older axis below by a development *de novo* of vascular tissue directly from pith cells.

In substance, these experiments of Ball and Wardlaw, although limited thus far to two angiospermous species only, give findings so closely comparable with those for the fern, *D. aristata*, that one may tentatively recognize them as being of general application. Together with the evidence already summarized, they indicate that the apical meristem is the morphogenetic region of the shoot which determines the pattern of differentiation below it. Another observation of Wardlaw's merits attention at this time. Initially the vascular cylinder was continuous. As leaves appeared, the stele became broken by leaf gaps. If those incipient leaves were punctured, as Wardlaw discovered for *Primula polyantha*, no leaf gaps appeared, and the stele was again continuous. In other words, initial stelar pattern is determined by the apical meristem, but it may be, and usually is, modified by the developing leaves. Thus the morphology of the stele reflects the phyllotactic pattern.

In a fourth type of operative procedure, apical meristems have been wounded by decapitations, by punctures, or by longitudinal splits with a view to understanding their regenerative capacities. The early work has

been reviewed by Ball (35); therefore it will not be considered in detail here. Suffice it to say that more extensive studies have been carried out in recent years with conclusions which increase our understanding of the potentialities of the shoot apex. Significant among the contributors have been Mirskaja (36), Pilkington (37), Snow & Snow (38, 39, 40), Snow (41), and Ball (35, 42 to 45). Mirskaja (36) split axillary buds of *Tradescantia guianensis* in a longitudinal plane and found that the halves grew into new shoots. Pilkington (37) not only split apices of *Vicia faba* and *Lupinus albus* longitudinally but also wounded them by decapitations and by pricks. Half-apices grew into normal shoots. Following decapitation and pricking, regeneration of apices was usual from undamaged portions of the old apex. Ball (42), in his early surgical work on *Lupinus albus*, divided the apex into four parts by two incisions made at right angles to each other. After an initial increase in size, each of the four parts usually developed into normal, mature axes showing only initial and temporary disturbances of the phyllotactic arrangement. If the apical segments were somewhat unequal, the smallest usually failed to develop, but the largest yielded normal leafy shoots.

In recent experiments (44), Ball has succeeded in dividing stem apices of *Lupinus* into six parts by three about equally spaced diametrical incisions. All but the smallest of these segments grew into small shoots, in which normal phyllotaxy was soon restored. Vascular continuity was maintained on the original peripheral flanks of the young developing shoots between the incisions as in the normal development of an intact apex. Progressively and gradually, the parenchyma lying parallel to the incisions and flanking these peripheral plates of vascular tissue became vascularized; eventually each growing and enlarging shoot acquired a siphonostele. And as leaf formation eventually returned to a normal phyllotactic sequence, so did the siphonostele show characteristic leaf gaps and become dictyostelic. Ball notes that the smaller segments which developed into shoots were initially protostelic; on further growth these fractional apices gradually increased in size and became siphonostelic.

As a by-product of isolation, removal, and attempted transplantations of central portions of apical meristems of *Lupinus albus*, Ball (35, 42) has extended the puncturing and pricking experiments of Pilkington and earlier workers. In no case were the transplantation studies successful. However, he reports that if the central portions of the apical meristem are removed from continuity with the periphery of that meristem by any one of the three methods referred to above, the flanking portions of the meristem characteristically initiate new apical meristems and eventually form shoots. As a result of these experiments, Ball points out how important it is to recognize that new initials are constituted from certain peripheral or flanking meristem cells when the original apical "initials" are destroyed. These new centers soon initiate apical meristems of new shoots, the number being determined by the number of centers formed. It would seem that the capacities of groups of these peripheral cells to assume the role of apical initials and to initiate a

new shoot are latent, so long as the original apical group continues to perpetuate itself, but become expressed when the original group is destroyed or isolated from its peripheral derivatives.

In those plants with large apical cells, e.g., certain ferns, the evidence of Wardlaw (27, 46) and Wetmore (unpublished) indicates that when the apical cell is punctured and certain adjacent cells probably killed as well, new buds form. Certainly the apical meristems of these new buds had their origin from peripheral portions of the original apical meristem. Whether they are precocious bud primordia, already ordained, or apical meristems formed *de novo* is not entirely clear. The evidence tends to point in the latter direction however. One wonders if it will be possible to discover whether Ball's findings in *Lupinus* (34, 45) will permit of similar interpretations.

Origin and development of leaves and buds.—It has been pointed out that leaf primordia originate on the flanks of apical meristems. In leptosporangiate ferns, the apical meristem is characteristically in the form of a small protruding cone situated on the apex of the rounded, distal region of the shoot—an arrangement rarely seen in other vascular plants. But in all types of vascular plants there exists a region at the extreme tip of the apical meristem which is free from leaf primordia. Near the base of the apical meristem, foliar primordia appear at approximately regular plastochrones [Askenasy (47)] or time intervals. Moreover, these primordia are also spatially arranged, thus establishing a phyllotactic system characteristic of the species. In some species, successive leaves appear singly and at a more or less constant angular relationship to neighboring leaves throughout the vegetative life of the plant. Thus in *Pisum sativum*, each leaf primordium arises in a position approximately 180° around the apex from its predecessor. By contrast, the primordia of *Syringa* or *Lonicera* originate in pairs, 180° apart on the apex, and each diametrically opposite pair is situated approximately at right angles to the immediately preceding opposite pair. Still others arise singly and are so spaced on the apical region that their bases seem to be arranged on a helix, for example, *Nicotiana* or *Brassica*. In many species, this helix seems to be of fixed physical character, such that the position of a new primordium can be closely predicted on the apex because of its uniform angular relation to its predecessor. But in still other species, for example *Helianthus annuus*, the helical pattern is a progressively and gradually changing one throughout the vegetative life of the plant, so that the position of a new primordium is more difficult to forecast.

In contemplation of the causal aspects of foliar inception, one faces at least three problems. (a) Why do leaf primordia originate at all? (b) Why do they arise in a definite and recognizable phyllotactic sequence? (c) To what factors do they owe their characteristic dorsiventral symmetry? Evidence exists which bears on these problems but a review of it indicates that full and adequate answers cannot yet be given.

Invariably, and at all stages of development, leaves are appendages borne on an axis. As previously indicated, Wardlaw (27, 28) first pointed out

that if very young leaf primordia are punctured or obliterated as fast as they are formed, the stem continues its growth and organization. This experimental finding at once sets aside possible adherence to the phyton concept, i.e., that the primary body of a vascular plant is an accumulation of units, each consisting of a leaf and its subjacent segment of stem. According to the phyton concept there is no stem as such, the apparent stem being no more than an aggregation of the basal parts of the several phytons. But such a concept is weakened by its failure to stand the test of experimentation.

In support of a hypothesis to account for the origin of leaf primordia, Schüepf (48, 49, 50) provided evidence that in *Lathyrus latifolius*, cell divisions were about equally distributed throughout the entire apical meristem. However, in the outer layers, anticlinal divisions predominated, whereas in the main body of the meristem divisions were haphazardly oriented. Consideration of his findings led Schüepf to believe that the peripheral layers would have to fold periodically if they were not to become too large for the internal body of meristematic cells which they covered. In his judgment, these folds were the leaf primordia. Extending his studies, Schüepf (51) concluded that the superficial layer of the apical meristem was pressing tightly against the body of the meristem below. Longitudinal splits in the apical region left no open gape or wound. Schneider (52) did not find experimental evidence to support Schüepf's belief; no tension appeared evident in the apical meristems of certain aquatics so long as he confined his investigations to the meristem proper, distal to the youngest leaf primordia; he inferred that Schüepf's findings may have resulted from splits made well down into the leaf-bearing portion of the axis. Ball (35) reports that no gaping followed incisions in apical meristems of *Lupinus albus*. These findings of Schneider and of Ball stand in contrast to evidence provided by Snow & Snow (53) who noticed that when gashes were made in the surface layers of the apices of *Euphorbia lathyris*, the wounds immediately gaped. This experience of the Snows has been common to many workers in the field [Wardlaw (54)]. To understand the seemingly conflicting conclusions of Schneider and Ball on the one hand and the observations of the Snows on the other, it may prove helpful to state from R. Snow's recent letter to one of the authors that he gets no gaping in apices which have not become partially desiccated during the experimental treatment. In any case there is no support in these findings for Schüepf's concept of the origin of leaves.

It must be admitted that as yet we have no clear alternative to Schüepf's interesting picture of the causal background for the origin of leaf primordia. Certain facts however have become clear. Leaf primordia are formed on the apical meristem and never elsewhere. Moreover, it has been ascertained that the entire meristem can give rise to these primordia even though ordinarily the topmost area of the meristem does not do so. Hence the suggestion has been put forward that the apical cell and its immediate derivatives constitute a zone or field of inhibitory influence; only at or just beyond this zone are leaf primordia normally formed. In this connection Wardlaw (23) has re-

ported that if young leaf primordia of a fern are isolated from the apical cell, they grow faster than normally. Moreover, when a very young primordium is isolated from its neighbors by longitudinal radial incisions, it also grows faster. That each primordium constitutes a growth center with a surrounding zone or field of inhibitory influence is thus indicated. Such experimental data of a morphogenetic nature prepare the way for relevant physiological investigations.

Wardlaw pictures the apical region of a leptosporangiate fern as comprised of a cone of meristematic tissue terminated distally by an apical cell and flanked by the large prismatic cells derived from it. All of these cells have been shown experimentally to be capable of producing leaf primordia. Ordinarily, however, because of the presence of a diffusible substance or substances originating in the region of the apical cell and diffusing downward from it, thus constituting the inhibitory field, leaf primordia can only arise in that region of the apical meristem which lies beyond the sphere of inhibition, that is, at a point or points on the diffusion gradient at which the concentration is sufficiently low. In the beginning, each leaf primordium consists of a group of superficial prismatic cells; active cell division pervades the group and shortly a centrally placed apical initial, the initial of the new leaf primordium, becomes apparent. A new growth center is thus started, round which a zone of inhibition develops. It is the continued, orderly production of new foliar growth centers which constitutes the phenomenon of phyllotaxis. Already in 1913, Schoute (55) had suggested the possibility of explaining phyllotaxis in terms of growth centers and their inhibitory fields. He held that in the process of leaf formation a leaf center was determined first and the leaf was organized around this center. A specific substance was thought to be produced by each growth center which had the effect of inhibiting the growth of other leaf primordia in proximity. Hence new primordia tend to form between and distal to pre-existing centers when the interfoliar spaces become sufficiently large. In Schoute's hypothesis, the extreme tip of the apex was also considered to act as a growth center with a surrounding inhibitory field. Richards (56) and Bünning (57) support the concept of growth centers as contributing to an explanation of organogenesis in plants.

Wardlaw (23) considers that Schoute's concept, with some modifications, applies aptly to the fern shoot apex, and has shown by an integrated series of experiments that it affords an adequate account of (a) the positions in which new leaf primordia arise, (b) the phyllotactic sequence, (c) the regulated development of leaves on the shoot, and (d) the inhibition of buds. We have, in short, a concept which affords an insight into morphogenetic processes at the apex and which lends itself to experimental examination.

Over more than the past hundred years the problems of leaf arrangement have been examined and reexamined. To one major group of workers the problems appeared to be, in the main, of a spatial or geometrical nature. Of this group, the most noteworthy have been Hofmeister (58), van Iterson

(59), and M. and R. Snow (38, 39, 40). Space does not permit a presentation of their ideas in detail. However, the experimental evidence of the Snows extends the ideas of Hofmeister and van Iterson and permits one to predict where a new leaf primordium will originate in relation to those already present. It will occur in the first space on the apex which attains a certain minimal width and is at a certain distance below the extreme tip of the shoot. The findings of the Snows are quite compatible with the field concept suggested by Schoute and developed by Wardlaw. For a new primordium will arise on the apical meristem (*a*) at a point of low concentration on the gradient of the inhibitory or regulatory substance produced at the apex of the shoot, and (*b*) in a space between two of the existing primordia which is more than wide enough to accommodate the sectors of their effective physiological fields. In fact, the phrase "available space" which has appeared so frequently in literature on phyllotaxis now acquires added significance for it may be doubted if "space" by itself has any causal value in morphogenesis. But space of adequate dimension may provide the necessary sustaining conditions for a morphogenetic process to take place [Wardlaw (23)]. Thus, the application of the field concept to problems of phyllotaxis has resulted in confirmation of the findings of M. and R. Snow and has given meaning to the concept of "available space" in leaf formation.

Of the other workers on phyllotaxis, some, such as Schüepf (60 to 63) and Hirmer (64) have suggested interpretations of leaf inception in relation to pre-existing internal structures. Consideration of the work of this group is not relevant to the discussion of the present article.

While the field concept provides a working hypothesis which seems to explain existing observational and experimental data, we do not yet know the nature of the diffusible substance or substances. The suggestion is made [Wardlaw (23)] that the physiological field probably results from the outward diffusion of growth-regulating substances from the apical cell and its adjacent segments, or by inference, from the growth centers of each primordium; but it must be pointed out that a field might also be organized as a result of the removal of substances by the apical cell from its adjacent cells and likewise by the growth center of any primordium from its surrounding cells. One can here, in contemplating the problems of leaf origin, advantageously turn to the considerations and interpretations of the field concept by animal embryologists [e.g., Huxley (65); Weiss (2); Needham (66), etc.].

While it is true that no concrete data exist which point to any single growth-regulator as the possible agent or as one of the possible agents in the organization of the apical region, there is some indirect and circumstantial evidence which suggests that indole-3-acetic acid may be directly or indirectly involved. Thus it is known that this compound inhibits the growth and development of buds. M. and R. Snow (67) proved that small amounts of auxin in lanolin (1/2,000) applied to individual leaf primordia promoted the growth of these primordia. It is well known that apices of vascular plants are established sources of auxin and that an over-all basipetal gradient is set

up in consequence [Avery, Burkholder & Creighton (68); Zimmerman (69); Guncel & Thimann (70)]. However, these studies have been made on extensive portions of terminal buds, each of which is comprised of the apical meristem and the leaf primordia and young leaves borne on the axis below it. To what degree the auxin diffusing from the bases of the axes in these studies was provided by apex and to what by the young leaves is unknown. Avery (71) in his study of development in tobacco leaves has shown that diffusible auxin was available from the bases of these leaves during the entire period of development, though the amount diminished as the leaves matured. Recently, Wetmore & Pratt (72) have carried out a systematic study of auxin production by apices and young leaves of the fern *Adiantum pedatum* L. A study of Table I indicates that the apex apparently provides less auxin

TABLE I
AUXIN DIFFUSED FROM APICES AND LEAVES OF *Adiantum pedatum*

APICES		
No. Apices	No. Leaf Primordia	Average Plant Units per Apex*
30	2 to 3	26.2
12	1	8.7
LEAVES		
Height of Leaves in cm.	No. Leaves	Average Plant Units per Leaf*
0.5 to 2.4	45	9.9
2.5 to 4.9	29	21.4
5.0 to 10.0	8	23.8
over 10	2	46.7

* The results of this study are comparative only; the amounts of auxin which diffuse from the apical region and from leaves of different ages are given in plant units after being corrected for sensitivity. A plant unit is the amount of auxin which will cause one degree of curvature of the *Avena* coleoptile in the standard *Avena* bioassay technique.

than do the young leaves on the same apex. Moreover, at successive stages of growth and development of leaves over 0.5 cm. long, the evidence appears conclusive that diffusible auxin can be demonstrated in significant amounts from the bases of the petioles. This auxin in the normal development must diffuse into the rhizome. While it is true that leaves beyond the primordial stage and less than 0.5 cm. in height have as yet not been assayed, the suggestion from the study thus far completed is that in *Adiantum pedatum* auxin is available from leaves at all stages of development. If this proves true, it

then appears clear that the leaves, at least during the entire period between their inception and their maturity, produce more auxin than they utilize, with the consequent result that the surplus will diffuse basipetally from the petiole into the rhizome. Whatever later investigators may prove for other plants, in *Adiantum pedatum* the evidence indicates strongly that the apical meristem of the shoot provides less net auxin to the basipetal diffusion stream than do the leaf primordia borne at or near the base of that apex or the older leaves borne lower on the axis. More studies are needed to give an adequate understanding of the auxin relations of apices and their associated leaves in diverse groups of plants. In order to permit an extension of studies on the distribution of auxin into the meristem proper, a new method of micro-assay of auxin is required. Its existence might provide information on the auxin relations of leaf inception. Also, the suggestion that leaves in all stages provide more auxin than the apex which bears them calls for an interpretation. Investigations at this level may provide information on the extent to which auxin is involved in morphogenetic processes. Moreover, the answer to the query as to the role auxin plays in morphogenesis, whether it is a primary substance in the organization of the apical and subapical regions, is dependent upon our acquiring the above information. Certainly, until we know whether auxin is directly involved in the inception of organs or is a product of their induction, or both, a confusion of cause and effect will remain.

A further note of caution is called for at this time. Not only is it impossible at the present time to know the part auxin plays in morphogenesis but it is quite conceivable that growth-regulating substances other than indole-3-acetic acid, and possibly effective at greater dilutions, may be involved in those very important initial phases of organ inception and tissue differentiation.

A consideration of the field concept leads to the view that the field with its growth center is probably asymmetrical. Whether on the side of a cone as in the leptosporangiate ferns, or on the surface of a more or less flattish mound as in most angiosperms, the physiological properties on the acroscopic and basiscopic sides are likely to be different. If inhibition is greater on the adaxial side, for example, the growth of the fern leaf primordium will be asymmetric and the resulting organ dorsiventral. With the query in mind as to the effect of eliminating the apical field, Wardlaw (23) isolated the area of the next potential leaf from this field by a tangential incision. When the growth center developed in this position, the resulting organ was radially organized and not dorsiventrally; in fact, it proved to be a bud. In other words, a bud arose in what is normally a leaf position; but in no case in his experimental studies was Wardlaw able to change what was visibly a leaf primordium into a bud.

As a new leaf primordium develops on the fern apical meristem, as indicated earlier, a panel of the prismatic meristematic cells above the leaf axil persists in an unaltered state; i.e., although the leaf primordium continues

growth, this area of cells remains inactive [Wardlaw (19, 20)]. In other cases the aggregation of cells does undergo certain divisions though these soon cease; the inhibited cell complex is then composed of smallish meristematic cells [Wardlaw (20); Garrison (21, 22)]. These detached meristems or bud rudiments are often recognizable far back on the axis, even after leaves have become fully grown. Each one, ordinarily inhibited from development, occupies initially a position on the axis just above the adaxial side of a leaf primordium. In fact, these bud rudiments or detached meristems are the residual interfoliar panels of meristematic tissue after the leaf primordia and their distended bases have been formed. Moreover, these panels may be variously displaced during the growth changes accompanying the expansion of the leaf bases, the elongation of the internodes, and the differentiation of foliar and cauline tissues. In fact, when buds develop in ferns, they may appear to have originated considerably above the axil of a leaf, even at times being attached to or a part of the abaxial side of the leaf petiole situated immediately above. In angiosperms, buds do not become so much displaced as in ferns.

While buds in ferns such as *Matteuccia*, *Onoclea*, and *Dryopteris* arise in positions above the leaf axils, a review of all the circumstances relating to their formation shows that at maturity they may be described with equal justice as occupying interfoliar positions [Wardlaw (20, p. 371)]. In exploratory studies on the stem apex, Wardlaw (23) destroyed, by puncturing, the five youngest leaf primordia around the base of the apical cone and also, by incisions or by punctures, the areas in which the next four potential leaves would appear. The apical cell was left undisturbed in each instance. The results proved interesting and in conformity with expectation; for on or near the edges of the punctured leaf primordia, in fact in the positions occupied by the detached meristems, leaf primordia were formed. These leaves, which were normal in their early development thus occupied bud positions, but were not in the normal phyllotactic order. This innovation of a leaf in a bud position, that is, of a dorsiventral organ in place of a radially organized structure, has added interest when one remembers that buds have been induced in areas which normally give rise to leaves (23). Wardlaw therefore infers that the apical meristem is totipotent; all of the tissue can be either leaf-producing or bud-producing. That which develops from any locus is determined by the position it occupies in relation to the physiological field surrounding it. "Incipient and developing organs are thus seen to have a functional aspect, for, on the hypothesis under consideration, the harmonious, orderly or regulated development of the leafy shoot can largely be referred to the physiological effects to which they give rise" (23, p. 126).

It may well prove true that this picture of the developing apex is oversimplified. Nor does evidence exist yet as to its applicability beyond certain ferns. In substance, however, as suggested by Wardlaw on the available evidence, it is as follows. In *Dryopteris*, the formation of leaf primordia is the initial morphogenetic expression of the apical meristem. The residual

interfoliar areas of meristem persisting after the formation of leaf and leaf-gap become the bud rudiments or detached meristems. It appears therefore that lateral buds are a later morphogenetic expression of the apical meristem than leaves. However, if the apical cell is punctured, leaf primordia continue to develop in the remaining apical meristem in regular phyllotactic sequence. If a young leaf primordium is isolated from the apical cell by a longitudinal incision, the development of such an isolated leaf may be slowed down, while bud development in the same segment becomes the noticeable feature. If, however, leaf development remains active, bud development is retarded. It seems evident that one effect of developing leaves is the inhibition of bud formation, though this inhibitive influence only persists while the leaves are young.

Despite the accumulation of data from extensive experimental studies of Wardlaw, Ball, etc., causal explanations of the formation of leaf and bud primordia are not yet available. Though the evidence points to an apical meristem which acts in somewhat of a regulatory way over leaf and bud formation, the biochemical agent (or agents), which is responsible for, or is correlated with, the formation of appendages, has not yet been identified. Indole-3-acetic acid, when directly applied in lanolin to a decapitated apex, can inhibit bud formation from primordia as can the physiological field of the apical meristem in the intact apex. This may be only accidental coincidence but it is interesting that the apex also produces an auxin which is essentially like indole-3-acetic acid in the *Avena* test. It will be an important step forward when the primary substance is identified which is causally related to or conceivably responsible for the incidence of leaf and bud primordia on the cauline apex.

No summary of the significance of the activities of the apical meristem would be complete without mentioning its role as an organizer tissue in relation to the initial blocking out of the stelar pattern of the stem behind it. Without the apex, no such organization occurs. It is evident from experimental evidence that some diffusible substance moves in polar direction back from the apical region. The identity of this substance is unknown, though again indirect support exists for a common identity with the auxin which is correlated with the origin of leaf and bud initials.

Tissue and organ cultures.—As a result of the now classical studies of Gautheret (73), Nobécourt (74), and White (75), it has become recognized that some plant tissues can be kept in an indefinitely active state of growth *in vitro*. Recently, Ball (76) demonstrated the possibility of growing whole plants *in vitro* from small portions of the apical region of certain angiosperms (*Lupinus*, *Tropaeolum*). While numerous workers have maintained cultures of apical portions of roots for long periods of time and have learned much about their nutritional requirements, the growth in controlled culture of whole plants from the terminal centimeters of roots has, so far as the authors know, not as yet been achieved.

Currently, Ball (personal communication, 1949) has been successful in

growing apparently normal young plants of *Sequoia sempervirens* from small portions of the apical region of a stem. Wetmore & Morel (77, 78) have demonstrated that it is possible for small pieces of apical meristem, from 250 down to 100 μ long and bearing two leaf primordia or even one leaf primordium to produce whole plants. In their work, successful growth of small apical pieces to normal plants obviously on the way to maturity has been achieved with the ferns *Adiantum pedatum*, *Osmunda cinnamomea*, *O. claytoniana* and *Pteridium aquilinum* var. *latiusculum*; with *Selaginella Willdenovii*; with *Equisetum hiemale*; and with such angiosperms as *Lupinus albus* and *Cucurbita texana*. In other words, one of the results of the surgical work is substantially confirmed by this cultural technique, namely, that the apical meristem, if properly nourished, is capable of producing a whole plant, shoot and root.

Initially, in their *in vitro* cultures, Wetmore & Morel employed as a basic medium that of Gautheret (79): Knop's mineral nutrient solution at half strength, supplemented by Berthelot's medium of minor elements, thiamine (1 p.p.m.), an auxin (usually α -naphthalene acetic acid at low concentration—0.001 to 0.1 p.p.m. varying with the sensitivity of the plant and the purpose of the experiment), and a carbohydrate source (usually 2 per cent dextrose, though sucrose or levulose seemed to be equally efficacious), all in agar of about 1 per cent concentration. Later, various modifications of the medium were made for specific studies, but only those resulting in observations of morphogenetic significance will be considered here. Thus, the addition of yeast extract in a concentration of 0.5 gm. per l., or of the Seitz-filtered milk from green coconuts (150 cc. per l.), to the regular Gautheret medium without added auxin proved beneficial to the growth of cultures of apical meristems. The best results were obtained consistently when yeast extract was used. Also, the effects of yeast extract were lasting in that, when apices of stems, after having been grown on a yeast extract medium, were transferred to a medium without it, the superior rate of growth found on this latter medium was maintained for several weeks after the transfer.

When yeast extract and green coconut milk in suitable concentrations are added to these cultures of apical meristems of different plants, the effects are striking. Salutary results have been reported from the addition of coconut milk to the medium of *in vitro* embryo cultures by several workers [van Overbeek *et al.*, (80)]. Caplin & Steward (81, 82) have also noted startling increases in the growth of carrot discs cultivated *in vitro* upon aerated liquid cultures containing 15 to 20 per cent of ripe coconut milk. That coconut milk has some factor or factors which does promote growth of apical regions in a variety of plants as well as the growth of certain so-called cambial callus cultures [Caplin & Steward (81, 82)] has, therefore, been clearly demonstrated. There is also some stimulatory substance or substances occurring in yeast extract effective in *in vitro* cultures of plants. Since the chemical nature of the growth substance in coconut milk is not evident as yet, its relation to that of yeast extract has of course not been established. Nor is

it clear that adding either of these mixtures to the medium upon which the explant is placed supplies the same substances which the parent plant itself would have provided. Pending the final resolution of this enigma of the nature of growth factors in coconut milk and yeast extract, the possibility must be continuously kept in mind that these factors may be supplied to the apical meristem by upward diffusion from some part or parts of the intact plant. In any case, the morphogenetic implications of the use of coconut milk and yeast extract in nutrient culture media are likely to remain open questions until the nature of their stimulation to growth is known.

The degree to which organic nitrogen should be or can be advantageously added to embryo, tissue, and organ cultures *in vitro* is not clear from the available literature. [For recent reports see Sanders & Burkholder (83), Riker & Gutsche (84), Nickell & Burkholder (85).] The basic medium containing inorganic nitrates as the only source of nitrogen proves adequate for growth of crown gall tissue (84), virus tumor tissue (85), and stem apices [Wetmore & Morel (77)]. Yet Riker & Gutsche indicate that nitrate, urea, alanine, aspartic acid, and glutamic acid deserve more attention, as their investigations suggest a possible role for them as "growth stimulating or encouraging substances" (84, p. 238) for crown gall callus of sunflower. Nickell & Burkholder (85, p. 546) also find evidence that aspartic acid was the only amino acid studied which could be employed effectively as the sole source of nitrogen for virus tumors of *Rumex*; it proved to be the "only compound, inorganic or organic, which approached nitrate in this respect." Riker & Gutsche found 11 amino acids which permitted no growth. When various amino acids were added to a complete medium containing nitrate nitrogen in such concentration as to provide good growth, the results indicated that the added amino acid was enough to effect generally a gradual inhibition of the expected good growth on the nitrate medium.

The above abbreviated account of recent literature provides no general knowledge of the morphogenetic effect of the addition of organic nitrogen to a basic medium for the growth of stem apices. Galston (86) reports that glutamine and arginine stimulated growth in asparagus stem tips, but he obtained no roots. Wetmore & Morel (77) have grown many stem apices of a variety of plants without any organic nitrogen in the medium. Upon the addition of casein hydrolysate to the basic medium for fern and *Selaginella* apices they obtained poor growth, a result which paralleled that reported by Riker & Gutsche for crown gall callus of the sunflower. Whether the addition of selected groups of amino acids to a medium would furnish results different from those obtained when they are added singly remains to be tried.

The apical meristem has long been looked at as a region of high protein synthesis [Priestley (87)]. That a terminal piece of apical meristem from 100 μ to 250 μ in length can grow *in vitro* into a well-organized plant with no nitrogen available in the medium other than nitrate also implies an active protein synthesis in the apex. Yet paper chromatographic studies [Allsopp (88)] of a wide variety of plants suggest a poor representation of free amino acids

in the apical regions. Whether his results might be expected if protein synthesis is high must remain conjectural for the present. It does not seem reasonable that enzymes are lacking for amino acid synthesis in the light of the excellent growth of apices on inorganic nitrogen salts though the general absence of free amino acids in Allsopp's work could be so interpreted. Certainly botanists will await with interest information on the metabolism of apical meristems. At present the indirect evidence provided from *in vitro* studies is too fragmentary and inconclusive to aid in the clarification of these problems. Rather it points to the need for carefully planned investigations, using the methods of the biochemist and physiologist, to supplement the limited approaches of the experimental morphologist.

The literature on vitamins provides little information on their role in morphogenesis. For recent reviews, Schopfer [(89), especially chapters 6 & 7], Thimann [(90), I, chapter 3], Bonner & Bonner [(91), chapter 6: 225-275] can be consulted, especially for the B-vitamins. In the culturing of stem apices, the work of Bonner (92) is suggestive; girdling experiments show clearly that thiamine moves from the mature leaves to the young leaves and to the apical meristems of the shoot and root. In the cultures of stem apices of Wetmore & Morel (77), thiamine has always been added as part of the basic medium. No systematic study of the significance of this vitamin in the activities of the apical meristem has been reported. Its possible relations to the genesis of leaf and bud primordia or to the differentiation of tissues behind the meristem are unknown.

The same general situation holds for the other B-vitamins and for the vitamins other than those in the B group, namely, that no comprehensive investigation relates to the roles of these substances in morphogenetic changes in the apical region of the shoot. Yet on a basic medium without them, the apical meristems have given rise to normal plants which, when transferred to soil, show no unusual characteristics. However, when a mixture of B-vitamins—thiamine, nicotinic acid, pyridoxin, and pantothenic acid (all at concentrations of 1 p.p.m.), folic acid (0.1 p.p.m.), biotin (0.01 p.p.m.), and inositol (100 p.p.m.)—is added to the basic medium with auxin at satisfactory concentration for good growth (0.01 p.p.m. for *Adiantum*), a better growth rate is obtained than without the vitamins, but not as good as with yeast extract or coconut milk. This has been true of all species tried, for example in plants as unrelated as *Adiantum pedatum* and *Selaginella Willdenovii*. The preliminary suggestion of an improved growth rate when the B-vitamin mixture is present is worthy of notice; its significance in terms of the possible value of some one or more of the vitamins as growth substances can only follow after much more investigation.

As indicated earlier, the basic medium for the growth of plant tissue used by Gautheret (73), requires auxin in low concentration; how low the concentration may be varies from species to species. It is interesting to note that the apical meristem of some species may produce a whole plant on a medium without auxin but at a slower rate. Wetmore & Morel (77) conclude that

whereas the apex of *Adiantum pedatum* does produce enough auxin for development if given time, the activity of the meristem is enhanced if a small supply of the hormone is added. Once the early leaves have become large enough to produce adequate auxin, there is a decreasing need for an extra supply in the medium. With leaves in all stages of development along the axis of a naturally growing plant, it seems likely that the diffusion of auxin from leaves into the stem may lessen the gradient from the apical region, with the result that the rate of development in this region may be more nearly that observed in cultures provided with auxin.

An important conclusion reached by Wetmore (78) results from the growth of apical meristems on several variations of the basic Gautheret medium. In all cases of modifications of this medium, whether by altering the concentrations of essential ingredients, e.g., auxin or sugar or both, or by the addition of such growth accelerators as coconut milk and yeast extract, he noted no significant variation in organization. With hundreds of plants of such species as *Adiantum pedatum* or *Selaginella Willdenovii* grown on diverse media, the essential differences proved to be in the rate and not in the pattern of development. These data point definitely to the fact that, in pure culture in varying media, the apical meristem sustains its own organization and continues to determine and control the characteristic organization, both external and internal, in the subjacent and maturing regions below it. In short, the fundamental morphogenetic processes of the species are unaltered even though the rate of growth and development may vary. This is an important result; it emphasizes the fact that the genetically controlled, developmental pattern is stable enough to withstand extensive variations in the nutritional environment.

It is important to note the contrast when concentrations of nutrient ingredients beyond the ordinary physiological range for normal development are used. For example, whereas with a concentration of auxin of 0.1 p.p.m. or 0.05 p.p.m. development of the tissues produced by an apex of *Equisetum* or *Syringa* is approximately normal, a concentration of 1 p.p.m. or 10 p.p.m. tends to yield a callus mass with abortive leaf production or none at all, and usually no buds or roots. Corresponding results are obtained when the medium contains higher concentrations than 0.5 gm. per l. of yeast extract, and appreciably higher than 150 cc. per l. of green coconut milk.

A summary of the results of the growth of apical meristems *in vitro* must therefore state the following conclusions. (a) The apical meristem of a shoot is the only known part of the plant which can control the organization of that shoot, thereby confirming the results of surgical manipulation of the apex, for only from an apical meristem adequately nourished is a whole plant grown. This conclusion is now supported by examples from such widely divergent major groups of vascular plants that there seems to be little question of its general importance. (b) An adequate medium to support such apical growth and organization calls for mineral nutrients, a carbohydrate source, and an adequate supply of auxin to expedite the rapid develop-

ment of leaf primordia. From the use of yeast extract and coconut milk there is evidence of the beneficial results of some unknown factor. Whether this factor exists in the plants whose apices grow better in its presence is as yet not known. The identification of the significant but unknown growth substance or substances in yeast extract and coconut milk may indeed prove an important step toward understanding more of the biochemistry of the stem apex. (c) Modifications of the medium used for the growth of apices, provided they lie within the range of concentrations observed in vascular plants, produce no appreciable changes in the characteristic developmental pattern; only the rate of development is changed.

In conclusion, we feel that the experimental evidence points to the apical meristem itself as an organized as well as an organizing region within which visible morphogenetic processes follow one another regularly and in order. Accord between histological and physiological organization is, however, not yet obvious. The appearance of leaf and bud primordia in the phyllotactic sequence which is characteristic of the plant indicates that genic expression in the apex is predictable and therefore open to experimentation. That auxin plays a part in the inception of leaf and bud is suggested by the experimental data. That it has an important role in the deposition of stelar pattern is even more strongly suggested. An understanding of the mechanism of auxin action in these developmental processes is, however, dependent upon greater information concerning the physiology of auxin in growth processes.

Experimental data indicate that developmental processes under the organizing action of the apical meristem can be modified in rate by various elements of the nutritive background—not only by carbohydrate supply—but also by growth substances.

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GAS DAMAGE TO PLANTS

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Gas damage to plants has been investigated for over 100 years. This subject has not been previously reviewed, therefore a number of old references will be cited, particularly those that carry important bibliographies. Sulfur dioxide has been the gas most extensively studied. More limited investigations have been made on other sulfur-containing gases as well as on the halogens and hydrogen halides, the oxides of nitrogen, ammonia, mercury vapor, and certain carbon compounds.

Early studies in Europe, largely in Germany, have been described in great detail in monographs by Haselhoff, Bredemann & Haselhoff (6), Haselhoff & Lindau (7), Schröder & Reuss (19), Sorauer (23), Stoklasa (26), and Wieler (48). Wislicenus edited a series of 11 papers (49) between 1896 and 1916 covering many phases of the smoke problem. The tenth paper in 1914, a summary of "Experimental Gas Injury," is of particular interest in this review. Haselhoff (5) published a brief summary of this literature in 1932. Most of the essential principles of smoke injury to vegetation as they are understood today were recognized in this early work. It was known that the gases in the smoke were much more important than the solid or liquid aerosols in causing injury to plants. Of the gases, hydrogen fluoride and perhaps silicon tetrafluoride were usually the most effective agents, followed in order by chlorine and sulfur dioxide. This order does not hold with all plants. Hydrogen chloride, ammonia, the oxides of nitrogen, hydrogen cyanide, and hydrogen sulfide required higher concentration to produce injury. Many other elements and compounds were also investigated to a limited extent and some were found to be very toxic, for example, bromine, iodine and hydrogen iodide, mercury vapor, ethylene, etc.

In the United States and Canada there has been great interest in gas damage to vegetation throughout the past 50 years. Extensive research has followed the periodic public concern for localized or general pollution. Swain (28) has reviewed the outstanding periodic developments of this century. The Donora incident (18) of October 1948, coupled with the investigation of atmospheric pollution which was already in progress at that time in the Los Angeles area (84, 85, 86, 88, 89) and a rather widespread concern over fluorine emanations, has resulted in another period of heightened interest in this subject and many new studies are being made and published currently. Also, a number of reviews of older work (13; 24, pp. 135, 142; 38) have appeared recently.

SULFUR DIOXIDE

The lesions caused by sulfur dioxide were accurately described by the earlier workers in this field as consisting of two types of injury on the leaves

(a) acute and (b) chlorotic or chronic markings. The acute injury consists of collapsed marginal or intercostal areas which at first have a dull water-soaked appearance, later drying and bleaching to an ivory color in most species, but in some becoming brown or brownish red. These lesions are caused by the rather sudden absorption of enough gas to kill the tissue. Chlorotic injury is a yellowing of the leaf which may progress slowly through stages of bleaching until most of the chlorophyll and carotinoids are destroyed and the interveinal portions of the leaf are nearly white. There is no collapse before this stage, but there may be some later, though the leaf is often abscised at this time. Chlorotic injury is caused by the sudden absorption of an amount of gas somewhat insufficient to cause acute injury or it may be caused by the absorption over a long period of time of sublethal amounts of the gas. In the latter case the buffer capacity of the leaf is eventually exceeded and the leaf ceases to function. A third type of injury, the so-called invisible injury, was postulated by Stoklasa (26, p. 101). Exposure to sulfur dioxide insufficient to cause any visible lesions on the leaves was nevertheless supposed to interfere with assimilation and reduce the growth or productivity of the plant. No experimental support for this theory has appeared, but on the contrary a large amount of experimental work has been done which demonstrates that this theory is entirely without foundation.

Environmental factors conducive to high physiological activity, such as adequate moisture and light and favorable growing conditions, were found to cause maximum toxicity of sulfur dioxide. Dormant plants and plants in the dark were much more resistant. Only the leaves of the plants were injured by low to moderate concentrations of the gas, such as were likely to occur in the field, and the principal avenue of entry was thought to be through the stomata. This explained the environmental factors. On the other hand, early methods of gas analysis were laborious and experimental fumigations were generally inadequate because of the practice of employing static gas mixtures for the fumigations, though Wislizenus (49) used limited air circulation. As a result, the toxicity of the gas appeared to be less than is actually the case.

Haselhoff (7, p. 109) thought that the sulfur dioxide attached itself to such compounds as aldehydes and sugars in the leaf and was held there. Then as these addition products were slowly split up, sulfurous acid or the sulfuric acid formed from it by oxidation caused injury to the leaf. He was uncertain as to whether the addition products themselves could cause injury. Noack (16) suggested that the sulfur dioxide inactivated the iron in the chloroplasts causing interference with its catalytic properties in assimilation. Then secondary changes affected the photochemical oxidative processes of the leaf so that bleaching and death of the cells resulted. Dorries (3a) suggested that acute sulfur dioxide injury could be diagnosed by an increased pheophytin content of the injured areas. The local acidity caused by the absorption of the gas could split magnesium from the chlorophyll molecule forming pheophytin. This reaction did not occur with chlorotic sulfur dioxide

injury, nor with a number of lesions similar in appearance to acute sulfur dioxide markings due to frost, weather, or disease.

The Selby Report.—The report of the Selby Smelter Commission (12) in 1915 was the most important and comprehensive of the early investigations of gas damage to plants in the United States. The report contains an extensive bibliography with abstracts. The Commission was concerned primarily with the effect of sulfur dioxide on the vegetation at the north end of San Francisco Bay. They also conducted extensive fumigation experiments and developed a method of air analysis for sulfur dioxide which permitted an evaluation of much of the earlier work and set the pattern for many of the later researches. Fumigation of vegetation was carried out by blowing continuously through a large celluloid-covered cabinet placed over the plants in the field a mixture of air and sulfur dioxide of sufficient volume so that the gas concentration, as it emerged, was not seriously reduced by absorption by the vegetation or cabinet. Correct toxic concentrations of the gas were thus established which were in general considerably lower than the earlier work had suggested. The rapid and reliable method of Marston and Wells was employed to determine the gas concentration both in the fumigation experiments and in the field. This method collected an air sample in a partially evacuated 20-liter bottle and determined the sulfur dioxide by iodine titration with a sensitivity of 0.1 p.p.m. Extensive fumigation experiments with barley were carried out. The lesions, consisting of mottled interveinal bleaching and "kill back" bleaching from blade tip, were illustrated with colored photographs. Photographs of sulfur dioxide injury to a number of broadleaf plants were also given.

Fumigation experiments at Vallejo, California, which produced severe bleaching when the barley was less than 15 cm. high, had little or no effect on the subsequent growth or yield. Similar fumigations between this time and the heading out stage reduced the yield 10 to 15 per cent. Reduction in yield of grain of 30 per cent was caused by "severe" injury when the grain was filling out. When fumigations, each of 10 min. duration, were applied daily throughout the growth period of the barley (90 treatments) the yield was reduced roughly in proportion to the gas concentration and the severity of injury. After 51 fumigations at 5 p.p.m., 50 to 60 per cent of the foliage was injured and after 90 fumigations, the "stalks were short and the heads were small." Ripening was delayed, and the yield of grain was reduced 45 per cent. A similar experiment in which 186 fumigations, each of 5 min. duration, were applied three times daily throughout the growth of the plants reduced the yield of grain as much as 51 per cent when the concentration reached 5 p.p.m. When 35 fumigations each of 2 min. duration were applied on 5 days in the "milk" stage, leaf destruction reached about 50 per cent with the 5 p.p.m. treatment and the yield was reduced 22 per cent. With lower concentrations, leaf destruction was less severe and the yield was reduced to a smaller extent. If the concentration did not exceed 1 or 2 p.p.m., there was little or no reduction of yield in most of these experiments.

For example, 198 fumigations at 2 p.p.m., each of 15 min. duration (4 per day) gave a full yield of grain, while 181 fumigations, each of 30 min. at 1 p.p.m. or 97 of 60 min. at 1 p.p.m. reduced the yield only 12 to 13 per cent. Parallel fumigation studies at Davis, California gave results consistent with those obtained at Vallejo.

In all cases, if there was no visible injury to the plants there was no reduction of yield of grain. These results were contrary to the theory of "invisible" injury and the possibility of the occurrence of such injury was considered to be remote. The converse was not necessarily true because "severe" injury in the early stages of growth or "slight" injury later had no measurable effect on yield.

American Smelting and Refining Co. studies.—O'Gara and Fleming carried out investigations in Salt Lake City from 1914 to 1922 parallel to those of the Selby Commission. Much of this work remained unpublished at the time of O'Gara's death, but Wells (47) in 1917 and Swain (27) in 1923 summarized part of it. The Selby work on the effect of sulfur dioxide fumigations on the yield of barley was confirmed. Similar yield studies on other grains, alfalfa, sugar beets, potatoes, tomatoes, red clover, and truck crops, again established a direct relationship between the extent of leaf destruction and the reduction in yield and again discredited the "invisible injury" theory.

O'Gara and Fleming were particularly interested in the relative susceptibility of different plants to sulfur dioxide injury and worked out relative factors for 350 species. The more important of these factors have recently been summarized (38). An important omission from these tables is cotton which has about the same sensitivity as alfalfa. Study of the effect of environmental conditions on the susceptibility of particular plants showed that high relative humidity, high light intensity, and temperature above 5°C. predisposed the plants to injury by the gas. The humidity effect was expressed by a mathematical equation (24, p. 142) which indicated a two-fold decrease in susceptibility between 100 per cent and 38 per cent relative humidity and an eight-fold decrease between 100 per cent and 10 per cent. Susceptibility was related to the opening of the stomata, as shown by the excellent photomicrographs of Loftfield (14), who did part of his work in their laboratory. O'Gara stated (17), as a law of gas injury that "the active portion of a gas concentration multiplied by the time through which it acts is constant" $[(C-1)t=K]$, where C is the actual concentration and 1 is a limiting concentration that can be endured indefinitely. Their equation for alfalfa was confirmed by later work (39). Finally they showed (27, 47) that gas damage to vegetation in the field resulting from the operation of a smelting plant could be greatly reduced or even eliminated by emitting the sulfur dioxide at a high temperature from a tall stack. Their work and subsequent studies of dispersion of gases from tall stacks have been discussed by Hill, Thomas & Abersold (10, 11, 42).

The investigations of the American Smelting and Refining Co. were

resumed in 1925 by Hill, Thomas and co-workers. They developed automatic analyzers for sulfur dioxide (30, 33, 34, 43, 44, 46) and for carbon dioxide (31, 41) in air which have been used extensively in laboratory and field studies. The sulfur dioxide "autometers" were based on the continuous absorption of the gas in distilled water containing hydrogen peroxide and the measurement of the electrolytic conductivity of the resulting acid solution. Carbon dioxide was absorbed in dilute alkali and the conductivity was measured as the hydroxide changed to carbonate.

Reduction in yield of alfalfa and other crops (9, 24, p. 142) due to leaf destruction by sulfur dioxide could be closely duplicated by clipping off with scissors an equal amount of leaf area from similar unfumigated plants. Evidently sulfur dioxide is not a systemic poison but its effects are confined to the local areas of injury which reduce the photosynthetic capacity of the plant by the amount of leaf tissue destroyed. Protracted fumigations (35a) with sublethal concentrations of sulfur dioxide had no effect on the yield of alfalfa if the sulfate supply to the roots was adequate. However, if the plants were sulfur-deficient, these fumigations increased the yield but not to the full extent expected of plants having an adequate sulfate supply to the roots.

Leaf destruction was dependent primarily on the rate and amount of gas absorbed by the leaves and only indirectly on the concentration of the gas (39). General leaf destruction vs. absorption equations were written for alfalfa from which it was deduced that the toxic dosage for the fresh mesophyll cells is about 540 p.p.m. (dry basis) of sulfur dioxide, if this amount of gas could be absorbed instantaneously. If absorbed over a prolonged period, the sulfur dioxide was partly oxidized to sulfate, which is only about 3 per cent as toxic as sulfite, and larger amounts of sulfur dioxide were needed to kill the cells. For this reason the sulfur content of alfalfa leaves could be built up to 1.5 to 2.0 per cent by protracted low concentration fumigations before the leaves became chlorotic, ceased to function, and were shed. In this case the pH of the system showed no change because the acid was neutralized by the organic bases in the leaf but the buffer capacity of the system was appreciably reduced (37). Repeating some of the work of Brooks (2) who studied the effect of sulfite solutions on water plants, it was found (38) that the toxicity of sulfite to the cells of *Elodea* and *Vaucheria* was nearly identical with the toxicity of sulfur dioxide to alfalfa mesophyll cells, suggesting a general protoplasmic reaction.

Studies of environmental factors gave results in agreement with those of other workers (24, p. 142). In addition, it was found that the amount of leaf destruction that could be produced by a standard fumigation applied to successive plots of alfalfa throughout a cloudless day rose from a very low value in the early morning after daybreak to a maximum at about 10 A.M. to noon, then fell off a little in the early afternoon, returning to a low value again in the late afternoon. The rate of absorption of sulfur dioxide paralleled the leaf destruction. The extent of stomatal openings at different times of

day offered an adequate explanation of the differences in absorption and injury except that in mid-afternoon there was not sufficient closure to explain fully the decreased leaf destruction. Possibly the gas united to some extent with the carbohydrates accumulated from photosynthesis and became less toxic. Katz (13) has described a somewhat similar "time of day" effect.

The effects of high and low concentrations of sulfur dioxide on photosynthesis and respiration (40, 41) of alfalfa and a number of other crops under field conditions or in large sand culture beds were measured using the automatic carbon dioxide analyzers. Parallel experiments were carried out simultaneously and independently in Canada by Katz *et al.* (13, 15, p. 393) with similar results. High concentrations (> 5 p.p.m.) inhibited assimilation almost immediately. If the fumigation caused extensive permanent acute leaf destruction, photosynthesis fell to a low level—even to zero—then increased in a few hours to correspond to the leaf tissue remaining uninjured. Subsequently, assimilation increased as the plant grew new leaves. If the high concentration was discontinued before appreciable acute injury occurred, photosynthesis regained a normal or greater-than-normal level in a few hours so that no appreciable over-all loss of carbon assimilation could be measured. Similarly, lower concentrations—as low as 0.45 to 0.60 p.p.m. for 3 or 4 hr.—reduced assimilation but if the treatment was discontinued before permanent injury occurred, there was little or no net loss of assimilation due to a considerable number of such treatments because of the slight post-fumigation stimulation of photosynthesis. With 0.3 p.p.m. or less there was no interference with assimilation until enough sulfate accumulated to cause chlorotic markings. The effect of the fumigations on respiration was less clearly defined. Katz (13) found that respiration was increased a little by the sulfur dioxide in some experiments and decreased in others.

Thomas *et al.* (4, 32, 35, 36) employed radiosulfur in fumigation experiments and found that the final fate of $S^{35}O_2$ in the plant after entry through the leaves was hardly distinguishable from that of $Na_2S^{35}O_4$ after entry through the roots, assuming of course that the former did not cause permanent leaf injury. In both cases the S^{35} was more or less rapidly changed to organic forms, depending on how much inactive sulfate was present in the plant along with the radiosulfate. In alfalfa leaves (37) the organic sulfur combinations were principally cystine and methionine as suggested by alkaline hydrolysis and established by paper chromatography (25). No methionine and only a trace of cystine could be detected in the free state although a small amount of glutathione appeared to be present. The sulfur amino acids, especially methionine, were therefore rapidly incorporated into the proteins. Evidence was presented (32, 38) to indicate that light was not necessary for the reduction of sulfate in the synthesis of the sulfur amino acids. Using radioautographs (32), it was demonstrated the S^{35} accumulated in the plant wherever proteins were most abundant, for example, in the

internal and external phloem and cambium of the roots and stems, in the mesophyll cells of the leaves, and especially in the fruit and seeds.

The Trail investigation.—In 1930, the International Joint Boundary Commission adjudicated a dispute based on alleged damage to vegetation in the State of Washington caused by sulfur dioxide from a large smelter in the Columbia River Valley at Trail, British Columbia. The case was subsequently placed under the jurisdiction of another international tribunal. The latter conducted its own investigations under the direction of its scientific advisors, Dean & Swain (3). The unique micrometeorology of the district was worked out by Hewson (8). Conditions were specified by the tribunal under which it was expected that the smelter could operate without causing injury to vegetation across the border.

Investigations bearing on this dispute carried out by the National Research Council of Canada were assembled in 1939 in a monograph (15) which is comparable in scope and importance to the Selby report. Katz (13) has recapitulated those sections of this monograph that relate to sulfur dioxide injury to plants and has compiled an excellent review of the literature with 122 references. Parallel studies of this situation by the United States Department of Agriculture remain largely unpublished.

The sulfur content of the leaves of plants in the Columbia River Valley (15, p. 104) was increased by exposure to sulfur dioxide so that, for example, the four-year old needles of conifers near Trail contained 0.6 to 0.8 per cent sulfur while similar needles 40 miles down the river contained 0.15 to 0.20 per cent, whereas the usual normal content was about 0.10 per cent. The leaves of deciduous trees absorbed about the same percentage of sulfur dioxide in one year as the conifer needles absorbed in four. An important phase of this work consisted of a tree ring study by Lathe & McCallum (15, p. 174) who made borings in 10,000 conifers to determine retardation of growth caused by sulfur dioxide and thus to define the area affected by the gas. When remedial measures were applied, injured yellow pine showed recovery immediately but the more severely injured Douglas fir did not show complete recovery in six years. Katz & McCallum (15, p. 218) found that these trees were uninjured by 0.25 p.p.m. sulfur dioxide for 450 hr., but young trees were slightly injured by 0.28 p.p.m. in 44.5 hr. or by 0.78 p.p.m. in 8 hr. Some young trees were killed by 1.11 p.p.m. in 15 hr. All trees were much more resistant in the winter than in late spring and early summer when they were physiologically most active. Larch was the most susceptible conifer.

Katz, Ledingham & McCallum (15, p. 51) presented an excellent description of leaf injury caused by sulfur dioxide including many photographs, some in color, to depict the lesions on the leaves of conifers, alfalfa, and other species, together with various other lesions due to insects, disease, or weather which might be confused with sulfur dioxide injury. Many photomicrographs are included in this outstanding pictorial display of gas and disease lesions. It was shown that when sulfur dioxide concentrations as they were measured in the smelter area were duplicated in experimental fumigations

at Summerland, B. C., where the atmosphere was free of sulfur dioxide, the lesions on the plants were similar in appearance, time of occurrence, and extent of injury to those in the smelter area.

Katz & Ledingham (15, p. 262) described the influence of environmental factors on susceptibility of conifers, deciduous trees, and crop plants to sulfur dioxide injury. They also studied extensively the relationships (15, p. 332) between leaf destruction, growth of alfalfa and barley, and the behavior of stomata (15, p. 298) with results in agreement with those of other investigators. In addition, many chemical analyses of wheat, barley and alfalfa for nitrogen fractions and carbohydrates were made by Katz & Pasternack (15, p. 369) which showed that there was no effect on the protein but a significant decrease in acid-hydrolysable polysaccharides in leaves severely injured by the gas. When no visible injury was apparent, even after protracted fumigations with low concentrations, there was no change detectable by chemical analysis in the proteins or carbohydrates of the leaves. These same conclusions have been extended to wheat by the work of Swain & Johnson (29) who conducted their experiments in controlled-environment chambers at Stanford. These authors, as well as Katz (13, 15, pp. 332, 369), Thomas *et al.* (35, 35a, 37, 40), and Setterstrom & Zimmerman (22) concluded that their studies on yield and chemical composition of plants and carbon dioxide assimilation during fumigations with sublethal concentrations of sulfur dioxide definitely disproved Stoklasa's theory of "invisible injury."

Brisley & Jones (1) conducted 278 fumigations of wheat plots during a five-year study in Southern Arizona to determine the effect of leaf destruction by sulfur dioxide on the yield of wheat. The leaf area actually destroyed by the fumigation was measured and expressed as a percentage of the total area developed by the control plants at maturity, including all the dead and discarded leaves. Because repeated severe fumigations inhibited tillering, the "area lost by treatment" was also estimated. This was done by counting the number of heads produced by the treated plants in relation to the number of the control plants since it was found that the leaf area of a wheat plant was proportional to the number of its heads. This method of expressing the results was quite different from that used by Thomas *et al.* (24, p. 145) who estimated the leaf destruction as a percentage of functional area at the time of fumigation.

Brisley & Jones found linear relationships between yield of grain and leaf area destroyed plus area inhibited for both single and multiple fumigations. The regression line for a single fumigation on a crop indicated that 60 per cent leaf area destruction would reduce the yield to 64 per cent of the control. Greater leaf destruction than about 60 per cent was not possible in a single fumigation because not more than about 60 per cent of the total foliage produced by a wheat plant during its life was green and functioning at any one time. When a crop was given two or three fumigations, separated by considerable time intervals, 60 per cent leaf area destruction plus area

inhibited reduced the yield to only 82 per cent of the control. Several reasons were given to account for the different slopes of the single and multiple fumigation regression lines. One factor appeared to be the poor growing season when most of the single fumigations were carried out. It was tentatively concluded that the more thrifty the crop, the less the damage sustained by the action of sulfur dioxide. Further work is needed to define these relationships between single and multiple fumigations.

In contrast with the results of the Selby Smelter Commission (12) and of Thomas *et al.* (24, p. 145) who found that fumigations between the boot and milk stages of growth of the small grains were more effective in reducing yield than at earlier or later stages, the data of Brisley & Jones do not indicate any definite stage-of-growth effect. However, if the leaf destruction values of the latter data for single fumigations are expressed as percentages of the functioning area at the time of fumigation, there is no appreciable reduction in yield due to extensive leaf injury until the sixth week of growth (boot stage). Then the yield falls to a minimum constant value between the blossoming stage and the ripening stage due to a given amount of leaf destruction. The treatments were evidently not carried far enough into the ripening stage for the lessened effect on yield of the fumigations to be observed in the "dough" stage.

Boyce Thompson Institute.—From 1934 to 1940, Zimmerman, Setterstrom and associates conducted a comprehensive investigation of sulfur dioxide effects on vegetation under different environmental conditions (20, 21, 22, 50), and these studies were recently summarized by Zimmerman (24, p. 135; 46). Experimental fumigations were designed so that the interactions of a number of variables such as water and nutrient supply to the plant, light, humidity, temperature, concentration, previous history, etc., on leaf destruction and yield could be calculated statistically.

Though sulfur dioxide was found (20) in the air at the Institute, 15 miles north of New York City, 62 per cent of the time in concentrations ranging from 0.01 to 0.75 p.p.m., no evidence has appeared that the vegetation growing at the Institute was ever damaged by the gas. In fumigations with 0.10 or 0.20 p.p.m. for periods up to 25 days alfalfa was uninjured; but with 0.40 p.p.m. foliar injury occurred in 7 hr. to both alfalfa and buckwheat. The protracted low concentration fumigations of alfalfa caused no decrease in yield under any environmental condition. A significant increase in yield was caused by such fumigation of sulfur-deficient plants, being most evident when the water supply was somewhat deficient and when the plants were growing rapidly under favorable light.

Resistance to sulfur dioxide injury was greater at temperatures below 40°F. than at higher temperatures in agreement with earlier observations. If the soil was dry enough to cause even slight wilting, resistance was markedly increased but with more soil moisture susceptibility was practically constant over a wide range of soil moisture. The degree of turgidity of the plants was thus very important. For example, turgid tomato leaves were severely

injured by 4 p.p.m. in 5 hr., whereas slightly wilted leaves were uninjured. Plants grown on poor soil were more easily injured than plants grown on good soil. Plants grown at low light intensity were more susceptible than plants grown in full sunlight. However, plants with the same previous history showed increased susceptibility with increased light intensity. Young plants were less susceptible than older plants and middle aged leaves were the most easily injured. The differences were probably due largely to variations of stomatal number, size, and function. Factors that did not affect susceptibility appreciably were: sulfur supply to the roots, unless sulfur was deficient in which case the sulfur dioxide increased growth rate; moisture on the leaves; previous fumigations; and small differences of relative humidity.

FLUORINE COMPOUNDS

Hydrogen fluoride appears to be a very toxic gas to certain plants but much less toxic to others. In recent years it has been the subject of a number of intensive studies by several experiment stations and public and private research laboratories. A very important consideration arises from the fact that forage which has absorbed a considerable amount of fluorine may cause fluorosis in cattle or sheep without showing any apparent lesions on the foliage (53, 58). Serious economic damage has been ascribed to this fluorosis (51). It has been claimed (53, 66) that forage containing more than 25 to 50 p.p.m. fluorine can cause fluorosis if fed for a sufficient length of time.

Early German investigators described fluorine damage to vegetation near industrial plants manufacturing metals, superphosphates, and ceramics. The European literature was reviewed to 1932 by Haselhoff (5, 6) and to 1941 by Romell (73). The fluorine is derived from the fluxes or impurities in the raw materials in these processes and the evolution of the fluorine therefore varies with the composition of the raw material. In the electrolytic aluminum industry a small amount of silicon tetrafluoride may be evolved at the anodes of the cryolite bath. The cryolite itself is also somewhat volatile. These gases may partially hydrolyze in the air to form hydrogen fluoride. In superphosphate factories the hydrogen fluoride arises from the action of acid on fluorides in the phosphate rock but with ceramics and other high temperature processes the reactions are not so obvious. Possibly acid derived from the sulfur compounds in the fuel attacks the fluorides liberating hydrogen fluoride (71) or perhaps silica is the liberating agent; possibly hydrogen fluoride and silicon tetrafluoride are both formed. No critical comparison of the toxicities of these two gases has been made, nor have they been determined separately in the atmosphere. The following discussion refers consistently to hydrogen fluoride although the gas may have been silicon tetrafluoride or fluosilicic acid in some cases. Elemental fluorine is probably too reactive to be present in any of these gases. The word "fluorine" in this review never refers to the free element without the specific designation "elemental fluorine."

Description of lesions.—The lesions due to hydrogen fluoride are somewhat similar in appearance to acute sulfur dioxide markings but their location on the leaf is often different. The descriptions of the lesions in recent literature agree well with the older observations. Haselhoff (7, p. 260) fumigated plum, cherry, oak, and chestnut with hydrogen fluoride. In the plum, dark brown spots and brown marginal lesions on the leaves were produced. In oak leaves, the injury started at the edge as sharply defined marginal spots. In the chestnut, the discoloration was light brown and the lesions appeared only as dark jagged lines on both sides of midrib and principal veins. The fig and apricot (59), peach (62), and prune (65) developed leaf lesions like the plum except that they were sometimes buff or ivory in color. Fluorine injury to walnut, lilac, blackberry, roses, and hydrangea (59) was also similar. The boundaries of these lesions were often sharp and the injured tissues might fall out after a few weeks leaving an irregular border or a hole. When the lesions were first formed they were pale green or gray water soaked areas, sometimes surrounded by a reddish brown border which subsequently faded. New freshly collapsed spots have been observed developing on the leaves of peach, apricot, and prune trees each day for over a week following a fumigation of moderate intensity (75). The first lesions on the peach leaves often did not appear until two to four days or more after the fumigation. On the peach also, some of the tender terminal shoots were pinched off a few inches from the end by the collapse of a short section of the stem. Brede-mann & Radeloff (55) found that hydrogen fluoride could be absorbed in the bark but not in the wood, causing the twigs to wither. They found no translocation of the fluoride to new growth. These authors (54) discuss the diagnosis of fluorine injury, describing gross symptoms like those already mentioned, which they consider need to be confirmed by chemical tests for fluorine, before the diagnosis is established. In other broad leaved plants (75) such as spinach and beet, large areas are often injured on one part of the leaf while the remainder appears to be uninjured. These areas may extend across large veins and even the midrib, a pattern seldom seen in sulfur dioxide lesions. Daines and associates (46) found that sweet potato was particularly sensitive to hydrogen fluoride.

Fir and pine needles (7, p. 260; 46) developed yellow tips which collapsed and turned reddish brown. Many other observations on evergreens agree with this description. According to Miller (46), the new growth of ponderosa pine needles may be shortened to one-fourth of normal length if the exposure is severe and the needles may be shed in one to two years instead of the usual five or six years.

Corn is particularly prone to chlorotic markings if the fumigations are insufficient to cause immediate collapse (75). A general mottled appearance may be evident on the leaves. In some cases tiny dark spots of water-soaked collapsed tissue have been seen which subsequently cleared up leaving small chlorotic areas. With higher concentrations, acute markings developed which were marginal and interveinal as well as at the tip of the blade. Similar acute

markings were produced on wheat, oats, barley and rye, but flax was nearly always injured across the tips of the serrations. Injury to the small grains caused by low concentrations was generally at the tip.

In the gladiolus the markings appeared at the tip of the blade and gradually extended down the leaf destroying an increasing area. The first visible symptom (60, 65) was a gray green collapse of the tissue at the leaf tip usually accompanied by a brownish streaking immediately below the lesions. The veins in this region, as well as the interveinal areas were killed. The lesions changed to a reddish brown, tan, or ivory color often with a dark brown border in the different varieties. They were referred to by Johnson *et al.* (60, 65) and others as "leaf scorch." Sometimes islands of marked tissues were found surrounded by healthy tissues, but this did not usually occur except with fumigations that could cause acute injury within a few hours. With more slowly developing lesions the injured area extended gradually and rather uniformly down the blade though the boundary might be somewhat irregular. Flower bracts might develop "marginal scorch" in a severe fumigation. The petals were sometimes injured also. Photographs of typical fluorine injury are given by de Ong (59) for apricot, by Leone *et al.* (62) and Daines *et al.* (46) for peach and tomato, by Zimmerman (46) for peach, by Miller *et al.* (65) for Italian prune, and by Johnson *et al.* (60) for gladiolus.

The injured leaves of fruit trees may be easily shed which in turn may interfere with fruit production. According to Zimmerman (24, p. 135), if half the leaves on a tree are injured by a fumigation, 25 to 50 per cent may be abscised in a few days. This is particularly true of apple, apricot, plum, prune, and peach leaves. De Ong (59) reported heavy defoliation of fig trees and 60 per cent defoliation of apricot trees near a fluorine-emitting plant in California on August 1, 1944, at which time the factory stopped operating. The yield of apricots was reduced in 1944 and 1945. Miller *et al.* (46) described an orchard located in Washington near a fluorine-emitting factory, in which half the leaves were shed by early August 1943, especially from the lower branches. These trees lost most of their fruit two or three weeks before harvest in 1943 and failed to set fruit in 1944 and 1945. Injury to the apical part of the fruit of an early plum was ascribed by Kotte (61) to fluorine as was similar injury to apples by Neger (67). Radeloff (70) found fluorine in the black skin at the apical end of fluorine-injured pears, but not in the green peel from their upper parts. Such direct injury to fruit has not been claimed in any recent literature.

Varietal sensitivity.—In the gladiolus there is a large range of sensitivity among the different varieties (58, 60). Some varieties may be killed in a field, while others nearby escape with only slight injury. It is an interesting fact that the varieties that are least sensitive usually absorb the most gas. For example, the Algonquin variety in a field plot was injured for a distance of only 7 per cent of its blade length back from the tip while the adjacent comparable Shirley Temple variety was injured 54 per cent (60). The Algonquin blades absorbed 611 p.p.m. fluorine while the Shirley Temple absorbed only

138 p.p.m. Compton & Remmert (58) found that within a variety, length of injury on the blade and fluorine concentration were closely correlated. There was also a marked concentration of fluorine in the tip of the blade. Under field conditions the tip 3 in. contained five to ten times as much fluorine as the second 3 in. portion of the leaf and sometimes 100 times as much as the basal 3 in. Oats showed this gradient without showing any injury. Translocation of fluorine to the tip was demonstrated by injury to leaf tips of Valaria and Picardy gladioli which had been sprayed a day or two earlier at the base with a weak solution of sodium fluoride or suspension of cryolite, the leaf tip being protected with cellophane during the spraying. Similar results were obtained by injecting fluoride solutions into the leaf base or by applying these solutions in lanolin paste near the leaf base. It has been noted that the seven to ten blades on a gladiolus plant differ considerably in fluorine uptake and extent of injury. Those leaves in the midportion of the plant have shown the greatest injury. Discrimination in the selection of leaf samples for analysis is therefore essential.

Large differences in ability to absorb and tolerate fluorine are also found between species. For example, according to Daines *et al.* (46), bean, spinach, marigold, and petunia remained quite uninjured after absorbing 200 to 500 p.p.m. in the leaves, whereas gladiolus and peach exhibited injury after absorbing as little as 30 to 50 p.p.m. Concentrations as high as 5000 p.p.m. have been observed in uninjured fumigated cotton leaves (75). Grasses (51, 58) likewise can absorb large amounts of fluorine without evident injury. Blakemore *et al.* (53) reported as much as 220 p.p.m. in forage. Churchill, Rowley & Martin (57) analyzed vegetation in the suburban and agricultural areas near Pittsburgh finding 9 to 269 p.p.m. in tree leaves and up to 53 p.p.m. in the grass. They also reported 85 to 295 p.p.m. in coal.

As already suggested, the accumulation of fluorine and the attendant injury at the tip of the blade or on the margin of the leaf is probably due principally to fluorine absorbed over the whole leaf surface and then translocated through the vascular system to those extremities. On the other hand, Romell (73) suggested that the high marginal concentration which caused injury was due to a "distortion of the diffusion field in the air around edges and protruding extremities." This distorted field presupposes a marked concentration gradient even at a distance from the exposed object, which is possible if the gas is sufficiently dilute and sufficiently rapidly absorbed by the object. Romell demonstrated this situation ingeniously by preparing leaf models of filter paper impregnated with dye sensitive to very dilute hydrogen fluoride or chloride. On exposure to the gas the tips and borders of the models became highly colored while the body of the paper was only slightly colored. Although the effect described by Romell may be a contributing factor in marginal injury, it should be noted that filter paper is not very convincing as a leaf model because it lacks the vascular system of the leaf.

Allmendinger *et al.* (52) have increased the fluorine resistance of gladiolus in the field by spraying the leaves with a nontoxic lime spray at one or two-

week intervals. In one experiment, tip injury was reduced from 9 per cent of the leaf area to less than 1 per cent while in another experiment, injury was reduced from 15 to 19 per cent to 2 to 4 per cent. When the leaves were washed with water after fumigation, 74 to 94 per cent of the total absorbed fluorine could be removed from the sprayed leaves, but only 11 to 25 per cent from the untreated leaves, indicating that the spray held most of the fluorine on the external surface. Dusts were less effective than sprays for this purpose.

Fumigation experiments.—Controlled fumigations with hydrogen fluoride are very difficult to carry out because of the avidity with which the gas is absorbed on cabinet and soil surfaces as well as by the leaves and because of the problems encountered in metering the gas. Equipment which is entirely satisfactory for sulfur dioxide may be inadequate for hydrogen fluoride. Wislicenus (49) placed the threshold marking concentration at about 2 p.p.m. Zimmerman (24, p. 135) used concentrations between 0.05 and several p.p.m. He suggested that 0.05 p.p.m. might be about the concentration that plants could tolerate indefinitely. He found that prune was slightly injured in 4 hr. by 0.07 p.p.m. and buckwheat, sweet potato and peach in 6.7 hr. by 0.085 p.p.m. These plants and also gladiolus, crabgrass, corn, bean, and pine were consistently injured by 0.1 p.p.m. while tomato required 0.67 p.p.m. for 2.2 hours to cause slight injury. All these plants were severely injured by 1.48 p.p.m. in 2.2 hours. Daines *et al.* (46) used either a glass or vinylite chamber in which the air was changed once each minute. Sweet potato, white pine, peach, and gladiolus were injured by 0.01 to 0.10 p.p.m., tomato, catbriar, smartweed, crabgrass, sorrel, tobacco, begonia, and geranium by 0.30 to 0.40 p.p.m. or less, spinach, pepper, and corn by 0.40 to 0.50 p.p.m., while aster, poinsettia, ragweed, plantain, zinnia, marigold, and petunia tolerated nearly 1 p.p.m. All these results are fairly definite in suggesting that some plants are very sensitive to hydrogen fluoride while others are much more resistant.

Compton & R Emmert (58) in Oregon fumigated gladiolus in a large portable vinylite covered greenhouse using about 1.5 changes of air per minute. The gas concentrations studied ranged from 0.0001 to 0.014 p.p.m. with continuous exposures of from 22 hr. to 6 weeks. The most sensitive varieties showed appreciable injury in a few days at 0.001 to 0.002 p.p.m. and slight injury at still lower concentrations. The Picardy variety under field conditions had about 1 in. of the blade tip injured after 5 weeks exposure to 0.0001 p.p.m. These leaves then carried 148 p.p.m. fluorine in the tip 3 in. At 0.010 p.p.m. for 22 hours, the injury involved over 4 in. of the tips and the tip 3 inch section contained 279 p.p.m. fluorine. Control plots treated exactly like the fumigated plots except for omission of the hydrogen fluoride showed practically no injury and the blade tips contained only a few p.p.m. of fluorine. There is some analytical uncertainty involved in the determination of such highly dilute gases but the average concentration determined by gas analysis was said to be about 80 per cent of the value

expected from the amount of hydrogen fluoride added to the air stream.

Mechanism of the toxicity.—The mechanism of the toxic action which can cover a threshold range of at least one-hundredfold in the fluorine content of the tissues and one thousandfold in the gas concentrations in different species is not understood. Enzyme systems may be involved. For example, phosphatase (64, 68) and other enzymes such as enolase (76) and amylase (72) are known to be inhibited by fluorine. According to Warburg & Christian (76), these enzymes consist of a protein molecule activated by combination with ionic magnesium or a bivalent heavy metal. Fluorine can form complexes with these metals and render the enzymes reversibly inactive. Another process that can be inhibited by a sufficiently high concentration of hydrogen fluoride is photosynthesis (75). In some species this function is quickly recovered if the fumigation is stopped before leaf destruction occurs. In other species, permanent inhibition can be produced without apparent leaf injury at first, but it has been noted in these treatments that leaf destruction develops gradually over a period of more than two weeks until eventually it corresponds with the decrease in assimilation rate. Wilson (77) found that fluoride in the leaves of tobacco increased the "bound" water, but whether or not this observation has any bearing on fluoride toxicity has not been determined.

Fluorine in the air.—Atmospheric fluorine analyses have been made near fluorine-emitting plants in Washington and Oregon but only a few have been published. Miller and associates (46) attributed the ponderosa pine blight near Spokane to some extent to fluorine gas injury from fluorine-emitting factories. Needle analyses in the area showed fluorine values ranging from 129 p.p.m. in the 1949 needles to 462 p.p.m. in the 1946 needles whereas outside this area the values ranged from 2 to 4 p.p.m. Air analyses near the place of maximum damage where many trees have been killed and others seriously defoliated ranged from zero to 0.064 p.p.m. fluorine. Largent (24, p. 129) quoted analyses of air near a superphosphate plant showing gaseous fluoride ranging from zero to 0.029 p.p.m. Kehoe (24, p. 115) states that systematic analyses of the air of Cincinnati from 1947 to 1949 gave average fluorine values from 0.003 to 0.006 p.p.m. Neither Largent nor Kehoe considered the question of leaf damage. Near Fort William, Scotland (51) where fluorosis of cattle and sheep occurred, total fluorine in the atmosphere ranged from 0.025 to 0.270 p.p.m. and gaseous fluorine from 0.009 to 0.140 p.p.m., at a distance downwind from an aluminum factory of 100 yards to 1 mile. Grass and soil samples ranged from normal (5 to 10 p.p.m.) to over 1000 p.p.m. Leaf lesions on vegetation, if present, were not referred to.

Fluoride from the soil.—MacIntire and associates (63) have studied the fluorine problem for 20 years in Tennessee and have contributed a great deal to our knowledge of the behavior of the element in the soil and its uptake by plants, as well as to analytical methods for its determination. They concluded that fluorine was not taken up by the tops of plants in amounts greater than a few parts per million if the soil was well limed. Acid soils

might yield plants with higher concentrations in both tops and roots, but liming the soil corrected this condition. In general, if an appreciable amount of fluorine was found in the tops, it represented deposition as dust or absorption as gas from the atmosphere.

The lesions caused by a soluble fluoride in the soil are quite similar to those caused by hydrogen fluoride. The New Jersey Experiment Station (62) found that the patterns of marginal injury for peach, buckwheat, and tomato due to fluoride in water cultures were nearly identical in all three species, but tomato required a considerably longer time for the symptoms to develop. The peach and buckwheat required 10 to 13 days to produce moderate injury at 25 p.p.m. in the nutrient solution, 261 and 533 p.p.m. being absorbed into the leaves, whereas the tomato required 27 days for symptoms to appear at 50 p.p.m. and 48 days at 25 p.p.m., at the same time absorbing 379 and 277 p.p.m. respectively into the leaves. Soluble fluoride added to soil became rather insoluble and therefore less toxic. Toxicity was decreased by raising the pH of the soil or by adding phosphate. The more fluoride fixed, the finer the soil texture. From these experiments, and others in which the plants were fumigated with hydrogen fluoride, the New Jersey group (46, 56) concluded that extent of injury to the tops and to the fluorine content of the leaves were about the same whether the element was added from the air or the soil. The two modes of entry could be distinguished readily by fluorine analyses of the leaves and roots. The latter were high in fluorine (1000 to 6000 p.p.m.) if the element entered from the soil but very low (<10 p.p.m.) if it entered through the leaves even though the leaves in the two cases carried about the same amount. When the plants were grown in nutrient solution containing fluoride they showed maximum injury when optimum concentrations of nitrogen and calcium and possibly phosphorus were also present. With larger or smaller amounts of these elements, injury was reduced. Plants growing in optimum nutrient solution also seemed to have the greatest susceptibility to injury by hydrogen fluoride but the quality of the nutrient supply had less effect if the fluorine was absorbed from the air rather than from the nutrient solution.

Evolution of fluorine compounds from plants.—Zimmerman & Hitchcock (78) reported that plants can evolve fluorine into the air after absorbing the element from the soil. Analysis of leaves indicated that there was a definite loss of fluorine two weeks after its absorption. Without quoting any figures, they state that the air in an isolated forest area of Pennsylvania contained more fluorine before leaf fall than afterwards; also that the fluorine content of the air inside the Boyce Thompson greenhouses in winter was higher than that of outside air. Similarly Brennan *et al.* (56) suggested that there might be a loss of fluorine to the atmosphere during the seven-day period following fumigation of tomatoes with hydrogen fluoride, but loss after absorption through the roots was not observed. Thomas *et al.* (75) obtained evidence by gas analysis of fluorine evolution from vegetation growing in sand cultures containing sodium fluoride in the nutrient solution. Loss

of fluorine to the atmosphere appears to be in conflict with the observation of Compton & Remmert (58) indicating measurable absorption by gladiolus of hydrogen fluoride at 0.0001 p.p.m. concentration. Further work is needed to clarify these relationships. Possibly owing to the marginal and tip accumulating action in the leaves that has been described, the two processes of absorption over the body of the leaf and evolution from the margin or tip can go on simultaneously.

OTHER HALOGENS AND HYDROGEN HALIDES

Hydrogen chloride.—The older European literature (5, 6, 7) discussed the action of hydrogen chloride on plants rather fully, but gave little attention to chlorine. In the early days of the Leblanc soda process, most of the hydrogen chloride from the treatment of salt with sulfuric acid was wasted into the atmosphere, causing extensive damage to plants near the factory. Between 1836 and 1863 scrubbers were installed at the various alkali works in England to remove at least 95 per cent of the hydrogen chloride in the stack gases and in 1874 the concentration in these gases was limited to 0.45 mg. per cubic meter, which eliminated crop damage. Hydrogen chloride is less toxic to plants than sulfur dioxide. The lesions are found principally on the margins or tips of the leaves but sometimes between the veins as well.

The fumigation experiments reviewed by Haselhoff indicate that the threshold marking concentrations are 50 to 100 p.p.m., but these values were obtained without adequate circulation of air over the plants. As much as a tenfold increase in the chlorine content of the leaves was observed. Limited experiments by Thomas and associates (75) on sugar beets suggest that the threshold marking concentration for a few hours' exposure is about 10 p.p.m.

Chlorine.—Chlorine injury to vegetation is somewhat similar to sulfur dioxide injury in being marginal and interveinal, but at least in some species such as sugar beets, the upper epidermis may also be attacked giving it a detached "silver leaf" appearance. This gas is more reactive to vegetation than sulfur dioxide (75). Only about one third to one half as high a concentration of chlorine as of sulfur dioxide was usually required to do equivalent injury. Thornton & Setterstrom (45) also found that chlorine was more toxic to plants than sulfur dioxide. Zimmerman (24, p. 135) fumigated many species with chlorine in the range from 0.46 to 4.67 p.p.m. for $\frac{1}{2}$ to 3 hr. Incipient injury occurred in $\frac{1}{2}$ hr. to beans and radish at 1.3 p.p.m. and to roses at 1.5 p.p.m. Buckwheat required 1 hr. at 0.46 p.p.m. and peach 3 hr. at 0.56 p.p.m. Fortunately, damage due to chlorine is rare and has usually resulted from isolated accidents. For example, Zimmerman reported two cases of chlorine injury near New York, one due to gas from a swimming pool that was heavily chlorinated and the other from an accident in a chemical plant. Stout (74) reported chlorine injury near a sewage disposal plant in California. The concentration of the chlorine in the air was not determined in any of these cases.

Iodine and hydrogen iodide.—Quantitative data (6, p. 215) on the other

halogens are lacking in the literature, but bromine and iodine appear to be very toxic. Unpublished data by Thomas and associates (75) indicate that iodine and hydrogen iodide were readily absorbed and accumulated in alfalfa. At about 0.1 p.p.m. (0.5 mg. per cubic meter HI or 0.8 mg. per cubic meter I_2) the leaves and stems absorbed 500 to 800 p.p.m. iodine in 18 hr. and the leaves were acutely marked resembling sulfur dioxide injury. At about 0.001 p.p.m. the plants absorbed 50 to 100 p.p.m. iodine in 8 days. Slight chlorosis of the leaves began to develop at this time. The accumulation factor from gas to vegetation was 5,000 to 100,000 in one to eight days.

NITROGEN OXIDES AND AMMONIA

Nitrogen oxides.—Nitric acid vapor (6, p. 187) and other nitrogen oxides can injure vegetation at about 25 p.p.m. Such injury has been observed occasionally close to factories making or using large amounts of nitric acid. Symptoms of injury include brown margins and brown to brownish black spots on the leaves and yellowing of the tips of needles. Blades of grain plants may assume a bright yellow color. However, there is little definite information in the literature about these gases. They are difficult to collect for analysis at high dilution. They are normally present in the atmosphere to the extent of 0.015 p.p.m. (6, p. 188). Maximum concentrations of 0.4 or 0.5 p.p.m. (24, pp. 67, 84) were observed near Los Angeles. They probably have considerable fertilizer value below the toxic range.

Ammonia.—Ammonia, like hydrogen chloride, is a gas of intermediate toxicity to plants (45). According to Zimmerman (24, p. 140), the pattern of injury was different from that caused by other gases. Nearly all parts of the leaf assumed "a cooked green appearance becoming brown upon drying." Slight marginal injury alone was sometimes observed. The variegated leaves of coleus "lost their brilliant color, appearing green thereafter." At 40 p.p.m. tomato, sunflower, buckwheat, and coleus were injured in 1 hr., at 16.6 p.p.m. they were slightly injured in 4 hr., and at 8.3 p.p.m. they were either uninjured or only slightly marked in 5 hr.

MERCURY VAPOR

The toxic action of mercury vapor on vegetation in an enclosed space has been known since 1797 when Deiman *et al.* (80) placed potted plants in a large bell jar with mercury. The leaves were spotted in 24 hr. and the plants were killed in a few days. This observation was confirmed by Boussingault (79). The subject was investigated thoroughly by Zimmerman & Crocker (82) at the Boyce Thompson Institute following the observation in a commercial greenhouse of injury to roses that was traced to the fact that mercuric chloride had been added to the soil to kill earthworms. Ratsek (81) observed a similar result at Cornell. The lesions consisted of brown spotting and abscission of the leaves, especially the older, active leaves. "The petals from partially opened buds were brown, the corollas of younger buds had turned brown and abscised without opening, stamens were killed, and the peduncles

were injured and were turned dark brown, or nearly black in places" (82).

It was shown that these lesions were caused by mercury vapor which could arise either from metallic mercury or even from mercury salts or organic mercurials mixed with soil. Indeed, mercury vapor could be found in the air and injury could be caused by mercuric chloride on a glass surface in an enclosed space with a plant, but not when the air was drawn through a tower of mercuric chloride before reaching the plant. Evidently, the organic matter in the soil or in the gases evolved from a plant could reduce mercury compounds to the metal. The higher the temperature, the greater the injury, as might be expected from the vapor pressure of mercury. No quantitative determinations of the mercury vapor content of the air were made. Since the vapor pressure at 25°C. is 0.00184 mm., saturated air would contain 2.4 p.p.m., mercury vapor. At 5°C., the concentration would be 0.4 p.p.m., at 10°C., 0.64 p.p.m., and at 40°C., 8.0 p.p.m. Little or no injury was noted at 10°C. or lower, but appreciable injury was observed at 25°C. or higher. After an exposure of three to eight days in a closed chamber with soil moistened with 1 per cent mercuric chloride, 317 to 4757 p.p.m. of mercury were recovered from the leaves of different plants. The lower values were from severely injured roses and the higher values from slightly injured tobacco, again illustrating the wide variation of different species in their response to different gases.

SMOG

Another agency that may be responsible for economically important damage to plants is smog. This term is ambiguous, but has come into use particularly in the Los Angeles area to describe the smoke and fog that characterize that basin. Fog is not a necessary component of the smog; in fact, more often than not, it is absent. Smog is generally distributed in urban and industrial areas, but economic plant damage due to smog has been reported only in the Los Angeles area, where, owing to the recent greatly increased industrialization, together with the low wind velocities of summer and autumn and large-scale, semi-permanent, non-diurnal temperature inversions (86), the industrial and urban wastes discharged into the atmosphere can sometimes reach concentrations sufficient to cause injury to vegetation (24, p. 148; 46, 82a, 83, 87). The injury has economic significance only in "heavy" smog when the inversion layer and the wind velocity are low and the concentration of the toxicants is raised materially above the usual "smog" level. The area affected by the smog may include the whole Los Angeles basin, extending from the metropolitan district east 65 miles to Redlands, south 125 miles to San Diego, or at least northwest 50 miles to Oxnard. Middleton and associates (87) do not report damage to economic crops in San Diego or Ventura Counties. The smog cloud may travel over the ocean to the west and south, then return to land at various places.

Went states (24, p. 148) that economic smog damage did not occur before 1942, but has increased steadily since then until in recent years, it has represented a serious loss. Middleton *et al.* (85) estimated these crop losses

in Los Angeles County amounted to \$480,000 in 1949, in addition to large losses claimed by the growers of cut flowers. They found that leaves of many crops, particularly endive, spinach, and Romaine lettuce, very readily developed leaf lesions that made the crops more or less unsaleable, always following days of extra heavy smog. Swiss chard, table beets, celery, oats, and alfalfa were also readily injured, but were usually a little more resistant than the first group. Tomato, barley, onion, parsley, radish, rhubarb, and turnip were still more resistant, while cabbage, cauliflower, broccoli, carrot, cantaloupe, cucumber, pumpkin, and squash were very resistant. *Gladiolus* (83) was also very resistant while *Poa Annua* (87) was the most sensitive plant observed. Other weeds, such as *Avena* sp., *Chenopodium album*, *C. murale*, *Galinsoga* sp., *Malva parviflora*, and *Sisymbrium* sp. were also rather sensitive and served as useful indicators of air pollution.

Smog gas injury.—Three types of leaf lesions have been recognized; namely, sulfur dioxide markings, smog-gas injury, and smog-fog damage (46, 83, 87). The first type has been found localized near certain industries. In this category, also, the possibility of injury by hydrogen fluoride or other special toxic agents is not precluded. Widespread "smog-gas" injury has occurred even in places remote from industry where toxic concentrations of sulfur dioxide or hydrogen fluoride or any other well-known toxic agents would be practically impossible. The pattern of "smog-gas" injury, as described by Middleton *et al.* (87), Thomas *et al.* (46), and Haagen-Smit *et al.* (83), is characteristic. Usually, the lower surface is attacked, thereby causing an oily appearance. A couple of days later, so-called "silver leaf" develops. The epidermis appears at first glance to be detached, but actually the subepidermal cells are collapsed, especially near the stomata, being replaced by large air pockets. The epidermis, which at first was not affected, soon dies. The upper surface opposite the silver leaf seems to be uninjured for a time, but it very soon becomes chlorotic. Then it usually collapses also, so that the markings extend all the way through the leaf. In extreme cases, or with very delicate leaves like the endive, the injured area may extend through the leaf immediately without "silver leaf." If the leaf has stomata on both sides, the silver leaf injury may appear first either on top or bottom or on both sides at once. Photographs of smog injury are given by Thomas *et al.* (46,) and by Middleton *et al.* (87).

Atmospheric pollution near Los Angeles is composed of a great many substances. The Stanford Research Institute (85, 88, 89) has identified at least 30 different organic and inorganic compounds. The Los Angeles Air Pollution Control workers (84) listed the concentrations of the pollutants of the atmosphere on clear days and very smoggy days. Average and maximum values of the gaseous pollutants are given in Table I. However, smog-gas injury appears to be due primarily to certain oxygenated organic compounds such as ozonides, peroxides, and acids (83). Sulfur dioxide is probably not directly involved. Indeed, injury seems to be enhanced if the sulfur dioxide is removed by a water spray as demonstrated by Peterson (46). Activated

carbon removes the toxic materials from the atmosphere as would be expected if they were organic vapors. The intriguing possibility of an antagonistic action between sulfur dioxide and the smog gases has been suggested (46). Such an antagonism could involve the reducing action in the leaves of the sulfur dioxide and the oxidizing action of the ozonides, etc. Presumably, reaction between these materials in the atmosphere is very slow.

A cooperative investigation of smog damage to plants (83, 84) to which the Los Angeles Air Pollution Control District, the University of California, and the California Institute of Technology contributed funds, personnel, and research facilities, has been made in the Earhart Laboratory at Pasadena. Plants were grown under controlled conditions of temperature, humid-

TABLE I
CONCENTRATION OF GASEOUS POLLUTANTS IN THE
LOS ANGELES ATMOSPHERE (84)

Pollutant	Average		Maximum Downtown
	Days of good visibility	Days of reduced visibility	
	P.p.m.	P.p.m.	P.p.m.
Sulfur dioxide	0.05	0.20	0.40
Formaldehyde	—	0.10	—
Aldehydes	0.07	0.20	0.40
Acrolein	—	Present	—
Organic acids	—	Present	—
Oxides of nitrogen	0.10	0.30	0.50
Ammonia	—	—	0.007
Oxidant	0.07	0.35	0.56
Sulfuric acid aerosol	0.0	0.02	0.036

ity, light intensity, and photoperiod. The fumigations were carried out in a room through which a constant stream of air was blown. This air had been made smog-free by passing it through an electrostatic precipitator and an activated carbon filter. Plants developed in this air without a trace of leaf damage. The gases to be investigated were introduced into the air stream at constant rates by metering devices. Only compounds that might be smog constituents were studied at first and only concentrations below 1 p.p.m. were of interest. The exposures were 5 hr. or less. It was found that most organic acids, aldehydes, chlorinated compounds, or saturated hydrocarbons caused no damage under these conditions. If there was injury at 1 p.p.m., it was not like smog damage but usually consisted of complete collapse of a portion of the leaf like that caused by strong acid gases. However, when vapors of the olefins were brought into contact with ozone, there was formed

a mixture of ozonides and other peroxidic compounds, aldehydes, and acids which caused typical "silver leaf" and other characteristic "smog" symptoms even at a concentration of 0.1 p.p.m. of ozone plus excess olefin for 1 hr. Lower concentrations caused injury with longer exposure. Oxidation of unsaturated hydrocarbons by nitrogen oxides in sunlight gave similar results. The greatest damage was caused by 1, n-olefins of a chain length of 5 and 6 carbon atoms: 1, n-pentene and 1, n-hexene. The position of the double bond in the end position is not essential for the action, as can be seen from the activity of 3, n-heptene as well as 4, n-nonene in the ozonization experiments. The composition of the oxygenated compounds derived either from the pure olefins or from the organic contaminants of the atmosphere which cause the leaf injury has not been definitely determined. There is, however, strong evidence that the active agent has to be found in the mixture of peroxydic compounds formed during ozonization, since mixtures of the ultimate decomposition products—the aldehydes and acids—failed to cause any damage in the concentration range used in the ozonization experiments. No tests have been made as yet with a pure ozonide or peroxide derived from a specific olefin. Haagen-Smit (82A) has discussed the chemistry of these oxidations and condensations, which are imperfectly understood at this time, in relation to smog damage.

In further support of the foregoing explanation of smog damage, the Los Angeles Air Pollution personnel (84) have been unsuccessful, in spite of thousands of attempts, in locating any appreciable amount of powerful phytotoxic substances in the effluent gases of any of the industries of the district. They therefore conclude that these materials are formed from more or less nontoxic substances after reaching the atmosphere.

Smog aerosols.—A third type of lesion may be designated "smog-fog" injury because it appears to be associated with only fog (46, 83, 84). It has been observed near Los Angeles following a heavy fog which caused considerable moisture to settle on the leaves. Small or large spots appeared on the upper surface and gradually extended through the leaf. Evidently, the tiny fog droplets coalesced into larger drops which slowly changed the color of the wetted area from bright to dull green. The injury was intensified as the leaf dried off. In some species, this coalescence did not occur and the whole upper surface was rather uniformly, but less vigorously, attacked. If the moist leaf surface was tested with indicator paper, a pH as low as 3.0 was often observed. The chemical composition of this residue has not been worked out. In the absence of experimental study, it would not be profitable to speculate as to its toxic constituents. Even if all the acidity represented by pH 3.0 were ascribed to sulfuric acid, it seems unlikely in view of the experiments of Wislicenus described later that this acid could be more than a partial contributor to the toxicity of the smog-fog.

Only limited attention has been devoted to the study of aerosol injury to vegetation. Wislicenus (49) sprayed small fir and pine trees with about 0.005 *N* solutions of sulfuric, hydrochloric, and fluosilicic acids, in the form

of very fine mists, for 5 to 8 min. at a time, five times daily, washing off the plants with water each evening. Spraying 459 times with hydrochloric acid caused no injury, though the chlorine content of the needles was increased 30-fold. Spraying 173 times with sulfuric acid caused slight injury, while continuous exposure to dense fumes of sulfuric acid made by passing air through "oleum" caused no injury in 14 days. Spraying 17 times with fluosilicic acid caused severe injury and 45 times killed the trees. It was suggested that the acid droplets themselves could not enter the stomata but that volatile acids might evaporate and enter the stomata as a gas. Experiments by the Selby Smelter Commission (12) and unpublished studies by O'Gara confirmed the fact that fine aerosols of sulfuric acid did not injure vegetation in several hours' exposure even at 100 to 500 p.p.m. in the air so long as a continuous stream of the aerosol was passed over the vegetation. When, however, the dense aerosol was allowed to stand in a closed container with the plants, the droplets agglomerated and settled on the leaves producing a leaf spot type of injury like that caused by "smog-fog." Experiments in Salt Lake City (46), carried out in collaboration with Paul D. Peterson of the Stauffer Chemical Co., also showed that dense acid aerosols (30 to 65 p.p.m.), through which the visibility was less than 3 feet, did not injure alfalfa or sugar beets in 4 hr. The particle size of this aerosol was one micron or less. When coarser aerosols—5 to 15 microns—were used, they settled on the leaves producing a definite "bloom" which could be readily seen. Under the microscope, the droplets appeared as perfect spheres resting on the leaf surfaces or the hairs. They persisted for days and even weeks. Under these circumstances no injury was produced on the leaves. However, if the leaf surface was wet with a drop of water or with a very fine spray of water, a spotted type of injury was soon produced due to the action of the acid on leaf tissues. Evidently the acid could not wet the leaf surface without the addition of liquid water.

CERTAIN CARBON AND SULFUR COMPOUNDS

Ethylene and other olefins.—Illuminating gas escaping into greenhouses either directly into the air or through the soil from broken underground pipes has been the cause of serious damage to plants. The lesions were quite different from those caused by the acid gases and consisted of epinasty, nutation, chlorosis, and abscission. Crocker, Zimmerman & Hitchcock (91, 95, 96, 103) attributed this damage to ethylene primarily but showed that hydrogen cyanide and carbon monoxide probably played a part also. Neljubow (99) observed that the epicotyls of the sweet pea developed abnormally in laboratory air, growing horizontally with a short thick habit. He was able to reproduce these effects by growing the plant in ethylene at 0.3 p.p.m. for several days and he suggested that the laboratory air contained ethylene from leaking gas connections. Knight & Crocker (97) and others (92, 94, 100) confirmed these observations on the behavior of ethylene at high dilution. They also found that the effectiveness of ethylene in causing epinasty

in the sweet pea was 1000, 2500, and 25,000 times that of acetylene, propylene, and carbon monoxide, respectively. Crocker *et al.* (91) found with tomato that the minimum concentrations to produce epinasty were 0.1, 50, 50, 500 and 50,000 p.p.m. for ethylene, acetylene, propylene, carbon monoxide, and butylene, respectively. The tomato can serve as an excellent test plant for detecting traces of ethylene in the air. They (91) obtained epinasty on buckwheat and sunflower with as little as 0.05 p.p.m. ethylene. Over half the 202 species tested showed no epinasty at all with 10 p.p.m. but nutation was inhibited. Recovery in normal air occurred at about the same rate as the epinasty developed and was complete in the younger leaves but the old leaves did not recover fully. Inhibition of nutation may be considered an anesthetic effect while epinasty is a stimulation of certain cells which, in the extreme case, can cause the premature destruction of the plant.

Illuminating gas produced epinasty, approximately in proportion to its ethylene content (95). Lily, narcissus, tulip, and hyacinth were treated for three days with an illuminating gas-air mixture containing 0.8 to 400 p.p.m. ethylene. Retardation in growth was noted at all concentrations without death or abscission of the leaves; curling, looping, and other distortions of the young leaves were produced with 3 p.p.m. or more, and young flower buds were killed except in the hyacinth; old leaves were not affected. Roses similarly treated (100, 103) developed chlorotic leaves which were dropped after two to five days, young flower buds opened and lost their petals within 24 hr., shoot elongation was inhibited, and an abnormal number of buds produced shoots after removal from the gas.

Carbon monoxide.—Carbon monoxide (102) was also found to be an anesthetic and stimulating gas that behaved like ethylene, except that concentrations at least 5000 times greater were required to produce the same effects. Carbon monoxide began to cause epinasty of the leaves of tomato at 500 p.p.m. At 10,000 p.p.m. for one to four days many species developed chlorosis and shed their leaves although others could withstand ten times this concentration. The effects resemble premature aging due to a stimulated metabolism. On the other hand, *Mimosa pudica* lost its sensitivity and coordination beginning 2 hr. after exposure to 1 per cent gas, thereby suggesting an anesthetic response. On removal of the plants from the gas there was rapid recovery, but an abnormally large number of side shoots developed, as noted for ethylene.

Hydrogen cyanide.—Illuminating gas seeping through the soil of a greenhouse is known to injure or kill the roots of plants. Hitchcock *et al.* (96) thought that this injury was due largely to hydrogen cyanide because if the gas, which might contain 200 to 300 p.p.m. cyanide, was scrubbed with alkali to remove this constituent, the toxicity was reduced by 95 per cent. The residual toxicity was thought to be due to phenols. When calcium or potassium cyanide was added to the soil in amount equivalent to the cyanide in the gas, similar injury resulted. Initial effects were noted with the addition of 4 to 10 mg. cyanide either as gas or salt to 500 gm. of soil. Natural gas

appears to be nearly free of substances that are toxic to the roots of the elm. Gustafson (93) maintained a continuous supply of 2 to 4 per cent natural gas on the roots of three American elm trees throughout the growing season of five successive years. Slight injury to the roots was noted but growth of the trees was not significantly effected.

When hydrogen cyanide is used as a fumigant in greenhouses and orchards, injury to vegetation sometimes results. Bartholomew *et al.* (90) fumigated Valencia orange trees with a standard treatment of about 1100 p.p.m. for 40 min. which is necessary to kill scale. Green fruits absorbed 5.4 times as much gas as mature fruits and were injured more readily. Injury to fruit and leaves was increased by irrigating the trees but not by spraying them with water and was greater in day than in night fumigations. Recoverable hydrogen cyanide was retained in leaves for 60 hr., in green fruit for 35 to 40 hr., and in mature fruit 20 to 25 hr.

Hydrogen sulfide.—Hydrogen sulfide is relatively nontoxic to vegetation, presumably due to its slight solubility in water. It has little practical significance as an air contaminant capable of injuring plants. McCallan *et al.* (98) observed no injury to 29 species fumigated with 40 p.p.m. for 5 hr. At 400 p.p.m. for 5 hr. some species but not all were injured. Its action was different from that of most other gases because it injured the young rapidly elongating tissues most severely. Scorching of the youngest leaves occurred first, then partial scorching of the somewhat older leaves, while the mature leaves were often uninjured. A few experiments with ethyl mercaptan (75) indicated toxicity at least as low as that of hydrogen sulfide but definite information on this gas is lacking.

Sulfur.—Finally, mention should be made of the injury caused by dusting elemental sulfur on foliage and fruit as a fungicide. Turrell (101) has treated this subject fully in connection with citrus, appending an extensive bibliography. He stated that injury occurred on both leaves and fruit in the humid coastal region of California but on fruit alone in the drier inland regions. Injury was confined to those parts of the plant that were exposed to the direct rays of the sun, particularly when air temperatures approached or exceeded 100°F. On the leaves the injury consisted of a necrosis ranging from small spots to extensive areas. On the fruit, it consisted of small white translucent spots which became pink in lemons or green in ripe oranges. Turrell showed that sulfur is volatilized appreciably at temperatures of 100°F. or higher in sunlight and that some of it is converted into hydrogen sulfide and sulfur dioxide, so that sulfur injury appeared to be due in part to these gases though many complex reactions were involved.

DISCUSSION

The phytotoxicities of the different gases seem to depend on: (a) absorbability, which is related to water solubility and reactivity with the tissues; (b) acidity or alkalinity; (c) oxidation or reduction reactions; (d) hormonal properties; and (e) toxicity of the element itself. Carbon monoxide, hydrogen

cyanide, and hydrogen sulfide exhibit comparatively low toxicities (50 p.p.m. or more required) because they are either rather insoluble in water or only slowly reactive with the tissues. The olefins are also rather insoluble, but ethylene appears to have powerful hormonal properties which make it effective in high dilution. Mercury vapor, while insoluble in water is evidently extremely reactive with organic matter, and the element itself is very toxic. The other gases are more soluble in water and some are extremely soluble so that they are avidly absorbed. The effect of acidity or alkalinity *per se* is shown by the intermediate toxicity (about 10 p.p.m.) of hydrogen chloride and ammonia. Possibly nitric acid behaves similarly. All these materials are merely neutralized by the buffer systems in the leaf, and because they are normal constituents of the leaf or are metabolized in the leaf, considerable accumulation is possible before they become toxic. The greater toxicity of chlorine and sulfur dioxide (about 1 p.p.m.) is due to their rapid oxidizing or reducing properties. Finally, the great toxicities of the fluorine and iodine compounds (about 0.1 p.p.m. or less) are due to their rapid absorption and the inherent toxicities of the elements themselves. Fluorine compounds exhibit a wide range of toxicities in different species, so that there is a corresponding wide range in sensitivity to hydrogen fluoride fumigation.

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PERMEABILITY

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INTRODUCTION

To the reviewer's knowledge, no notable new concepts have been outlined nor new techniques developed for the period July, 1948 to June, 1950, relating to permeability. However, there has been a rather marked shift in emphasis over the past several years and an attempt will be made to note and evaluate major trends. While many biological periodicals have been consulted, important omissions most probably will be noted and for these the reviewer apologizes in advance. The reviewer has arbitrarily omitted mention of preliminary short reports and abstracts, even though they may have appeared to contain material of considerable interest. In a field so difficult of interpretation as permeability, it is felt that nothing short of a complete report warrants critical review. A number of references to literature on permeability were included last year by Kopac (1) in his review of protoplasmic structure.

The years under review were marked by the loss of two men who made notable contributions to our basic knowledge of permeability problems. Leonor Michaelis carried out, in addition to his many other activities, the original detailed investigations of dried collodion membranes which have proven so useful as models for some cellular phenomena. August Krogh, likewise a scientist of varied achievements, spent many of his last years studying active ion uptake by whole animals as well as by cells and tissues. He pioneered in this work which has recently engaged the attention of an increasingly large number of competent investigators.

The reviewer recommends that the stimulating discussion in the first part of the review by Teorell (2) be re-read periodically by all students of permeability. Insofar as it is possible, the terms used in this review will conform to those defined by Teorell.

Teorell, in his 1949 review (2), stated that it was his impression that there was a considerable decrease in the number of published papers dealing with permeability, as compared with previous periods. This impression is shared by the present reviewer and is borne out by the listings of "Permeability" as related to living systems, in *Chemical Abstracts*, 32 items being listed in 1949 as contrasted to 46 in 1941 and 88 in 1937. This does not reflect any diminution in interest in problems of exchange of materials between living units and their environment, but rather a recognition that such processes are best studied in terms of other general activities of organisms such as assimilation and nutrition, rather than as phenomena, ends in themselves. Observations relating to permeability are no longer most apt to be found in papers using the specific term in the title. Thus it becomes even more

important to refer the reader to chapters dealing with secretion, mineral nutrition of plants, absorption, and structure of protoplasm, as well as to those reviewing activities of special organs such as kidney, skin, placenta, liver, and so forth. Also relating to the wide dispersion of papers containing material of interest to students of permeability is the fact that reviews of the subject tend more and more to reflect the special interests of the reviewers. The present discussion is no exception to the trend.

GENERAL COMMENTS

If a given substance can be shown to pass through a boundary, the boundary is said to be permeable to that substance. The term "permeability," in a strict sense, means just this property. Nothing is implied about the mechanisms involved in the transfer although, in the past, permeability studies have been used to argue for free diffusion in pores, diffusion by solubility in a phase boundary and so on.

In a general way, it can be said that much work on permeability in past years has been done from an "equilibrium" point of view, interest centering around considerations which assumed relatively static, inert conditions for the cell, especially the cell surface. This point of view was admirably summarized by Davson & Danielli some years ago (3). A cell or tissue was placed in a solution of, say glycerol, and either rates of penetration of glycerol or volume changes of the cells were measured. The results could then be described in terms of the usual diffusion equations and constants assigned, not always, unfortunately, with due regard for variables included in the "constants."

More recently, there has been an increasing tendency to recognize the living cell as an active part of an open system. Perhaps the point of view can be best explained by quoting from a paper by workers whose approach to problems of cell permeability is refreshingly untouched by traditional words (4). "... the dominating factor in cell permeability is not any inherent and constant physical property of the cell wall, like porosity or lipid-sieve structure, but a mechanism dependent on the supply of energy." The reviewer feels that the general philosophy expressed will continue to prove useful, provided, of course, that static aspects of the problems are not ignored.

Isotope tracer studies have emphasized some of the discrepancies in the use of the term "permeability." Prior to the advent of these agents, only total changes in amounts of substances in cells could be measured by direct analytical procedures, or inferred indirectly as with experiments on volume changes. With isotopes, net changes and exchanges may be measured. For example, in the frog sartorius, the permeability of the membrane for sodium ions (free diffusion) is probably rather low, while the permeability to sodium, as measured by isotope exchange, is high (5). The inference is that the membrane, in addition to allowing a slight leakage of sodium ions, also has a specific exchange system which may not appear in conductivity measure-

ments and which does not lead to a change in total amount of sodium within the fibers and hence would not appear in analytical chemical studies. Especially with isotope studies, it is becoming increasingly important to specify conditions precisely and to recognize whether changes in total concentration or only exchanges are involved before comparing results with those obtained by other methods. Needless to say, it is also important to guard against injurious effects of radiations when radioactive isotopes are used. No papers dealing with this problem have come to the attention of the reviewer. Numerous useful discussions on applications of tracers to biological problems may be found in a recent symposium (6).

For many years it has been recognized that some substances, added to the external environment, first combine specifically with the cell surface and then may penetrate, turning up, apparently unchanged, in the inner protoplasm. Since this appears to involve incorporation of the externally applied substance into the structure of the living cell, the process can be thought of as assimilation and the propriety of designating the cell to be permeable to the substance in question may be doubted. There is an increasing number of papers whose titles read "Uptake of . . ." rather than "Permeability to . . ." The case of phosphorus in yeast, as discussed by Spiegelman & Kamen (7), illustrates the situation. Tracer phosphate, added to the medium, is taken up by yeast in the presence of sugar. The process may be stopped by appropriate inhibitors. Kinetic analyses and isotope distributions among the various phosphate fractions lead to the conclusion that phosphate enters by combination at the cell surface, some of the entering material appearing soon inside the cell as inorganic phosphate. Thus orthophosphate, added to the medium under the proper conditions, appears as orthophosphate inside the cell and, according to definition, the cell is permeable to phosphate. On the other hand, a given molecule may have gone through many stages of identification with synthetic end products between the time it was first combined at the cell surface and the time it appeared in the interior as the same type of compound originally added to the medium. In a sense then, the cell never was permeable to orthophosphate; the compound appeared in the interior because it was assimilated, finally, and only incidentally, in its original chemical state. In similar fashion, calcium added to the medium is taken up by *Elodea* cells and then given off to form insoluble oxalates in the vacuole (8). By analogy with the phosphate story, it could be argued either that the cell was permeable to calcium or that the cell was impermeable to calcium but assimilated the element.

Ideally, it should be possible to discuss the uptake of materials by cells, or loss from them, either as a passive process taking place due to diffusion through a boundary or as an active process of assimilation and synthesis. The processes of the latter category must, of course, take place against a background of the simple driving forces of the former (chemical or electrochemical gradients) but involving such special mechanisms as to render the general definition of permeability almost useless. At the present time, how-

ever, the reviewer sees no alternative to adopting the very general definition of permeability, attempting to sort out mechanisms as work proceeds.

PERMEABILITY TO STRONG INORGANIC ELECTROLYTES

This heading is retained by the reviewer for want of a better designation, in spite of the probability that, in many instances, the penetration of cells by the ion-forming constituents of the medium may well be as non-ionized bound combinations. A discussion of exchange phenomena in general may be found in Ussing's review (9), and in the literature on salt uptake by plants (10). A monograph on ion binding in soils is informative and useful (11). The paper by Hodgkin & Katz (12) contains calculations regarding ionic conductance in the nerve membrane, and Katz assembles data on electrical characteristics of muscle and nerve (13). Danielli has written briefly on concentration of ions at interfaces (14). Nachmansohn (15) has reviewed problems of permeability as related to nerve function with special stress on movements of inorganic ions, acetylcholine and related substances, and the anticholinesterases.

Muscle and nerve.—Observations over the past several years, especially with radioactive isotopes, have confirmed the findings with analytical chemical methods that muscle and nerve are apparently permeable to all of the normal inorganic constituents of the environment (16). Sharp attention is thus focused on problems of specific ion transport mechanisms, in order to account for the normal ion distribution between cell and environment. Special interest has been aroused among electro-physiologists by the suggestion of Hodgkin & Katz (12) that the "overshoot" of the action potential may be explained in terms of marked alterations in permeability of the nerve axon to sodium.

Considerable attention has been paid to the sodium-potassium balance in muscle and nerve fibers (9). Levi & Ussing (5), after equilibrating frog sartorius muscles with Ringers' fluid containing Na^{24} or Cl^{38} , studied the time course of washing out of radioactive isotopes. Na^{24} left the muscle in a fashion suggesting a rapid removal from the interspaces and a slower loss from the fibers. Approximate half times for Na^{24} loss from fibers were 34 min. at 20°C . and 70 min. at 1°C . Cl^{38} apparently left the fibers at a faster rate. No effects of altered potassium concentration were noted on sodium or chloride movement. The authors, after consideration of energy requirements, conclude that Cl^{38} movement may be by simple ionic diffusion, Na^{24} most probably as specific ion exchange or transport in large part with small but unknown fractions of free ionic diffusion. Harris & Burn (17) have studied both uptake and loss of K^{42} and Na^{24} by sartorius muscles of the frog. Their paper contains an extensive treatment of methods of calculating permeability and diffusion constants in a system of interspaces and fibers. Average permeability values obtained were 3.4×10^{-3} cm. per hr.^{-1} for potassium and 5.2×10^{-4} cm. per hr.^{-1} for sodium. No effect of altered potassium concentration on either sodium or potassium permeability was noted.

Rothenberg (18) has reported on the uptake of Na^{24} , K^{42} , and Ca^{45} by squid giant fibers immersed in artificial sea water enriched with the appropriate elements. Uptake was measured by collecting extruded axoplasm after equilibration of the whole nerves and axons. "P" values calculated according to Krogh gave 1.25×10^{-3} cm. per hr. for potassium and 5.76×10^{-2} cm. per hr. for sodium as representative values. The precise significance of the constants is difficult to evaluate, however, due to an incomplete (10 per cent) exchange of K^{42} . Ca^{45} penetrated into the axons in a rather complicated fashion, first apparently accumulating and then partially leaving the fibers after longer immersion times. Electrical excitation increased the rate of Na^{24} penetration, as did x-ray irradiation. Anticholinesterases increase the rate of Na^{24} penetration and decrease that of K^{42} . Cocaine has no marked effect.

Keynes (18a) has reported on influx, outflux, and exchange constants for Na^{24} and K^{42} with Sepia axons. In resting nerve, the influx of Na^{24} is about three times that of K^{42} , and during activity, over 20 times. This provides direct evidence for the suggestion of Hodgkin & Katz (12) that sodium entry increases markedly, as compared to potassium, during excitation of nerve. It is perhaps worth noting that the ratio, influx Na /influx K , during activity, approaches the ratio for external concentrations of the respective ions.

Leakage of potassium from brain during anoxia has been noted by Dixon (19), from embryonic chick muscle during cooling by Wesson *et al.* (20) and from peripheral nerve by Fenn & Gerschman (21). The latter authors find a considerable increase in potassium loss during anoxia as compared to normal. Acetylcholine also increases potassium loss. Fischer (22) has found that potassium is lost and sodium is gained during atrophy of muscle, but only to a limited extent. In general, potassium loss parallels loss of extractable myosin.

An increased rate of potassium loss from muscle, nerve, and other systems, following exposure to anoxia and other conditions, has sometimes been interpreted as evidence for an increase in permeability to potassium. Great care should be exercised in making such judgments, since several factors are concerned with any rate of net movement of ions. Ions tend to move under the influence of a driving force defined as an electrochemical gradient (= chemical gradient and potential difference). In a normal resting muscle, potential differences balance chemical gradients nicely for potassium and chloride (2, 9). Any treatment tending, for example, to decrease the potential difference would automatically throw off the balance, resulting in a rapid net flow consistent with the chemical gradient. In addition, any alteration of a transport mechanism, for sodium for example, could cause net shifts in other ions according to their respective chemical gradients. It is thus very easy to have "false permeability changes" if any factor leading to normal distribution of ions is neglected. A change in driving force does not imply a change in permeability.

The interesting studies on sodium-potassium balance of insects have been

continued by Tobias (23). Shenk (24) has compared methods of chloride determination in muscle tissue and concludes that the usual HNO_3 digestion gives low values as compared to the Parr bomb method. Fleckenstein (25, 26) develops the novel suggestion that the primary source of energy for contraction of muscle resides in the osmotic pressure relationships of intra- and extra-cellular sodium and potassium, sodium normally being bound in an outer envelope. It is difficult to visualize the coupling of such a system to a contractile mechanism [cf. also (27)].

Feng & Liu (28) have investigated the effects of salts on amphibian nerve in an attempt to evaluate the permeability of the sheath to these agents. They conclude that the sheath is a real barrier to passage of inorganic components of the medium, a conclusion that is hotly contested by Lorente de N6 (29). The reviewer is of the opinion that the controversy illustrates very well the dangers of attempting to determine permeability to an agent by studying the physiological effects of the agent. The system under discussion by the several authors is very amenable to direct investigation.

While perhaps not of primary interest to the subject of permeability, attention should be drawn to the calculations of Hill (30) showing that diffusion, presumably of ions such as calcium, could be fast enough to account for the coupling of excitation and contraction in striated muscle fibers, provided that the whole cross section of the fiber need not be involved. In a later paper, however, Hill (31) concludes that during a single twitch the whole fiber does respond and hence there must be some inward transmission of excitation other than diffusion of ions.

As in prior periods, there have been numerous bioelectric studies, most of which are interpreted in terms of permeability to ions. The reader is referred to discussions of muscle, nerve, and bioelectric potentials for details. While membrane conductance and impedance measurements in general give direct evidence of permeability to ions, the reviewer feels that the usual resting potential studies can be related to permeability only with great caution. Wide variations in permeability may be demonstrated in artificial membranes with little or no change in concentration potential (32).

Multicellular membranes.—With the primary aim of studying active uptake and transport of ions, a series of investigations has been reported from the group in Copenhagen formerly headed by Krogh. Ussing (33) has reviewed much of this work, the major results of which will be mentioned under the heading ACCUMULATION AND ACTIVE TRANSPORT OF IONS. Ussing (34), using Na^{24} , shows that sodium flux from outside to inside of frog skin is much greater than in the reverse direction, the difference tending to be abolished by cyanide which causes a decrease of influx. Epinephrine action tends to equalize influx and outflux because of a differential increase in both. Barker-Jørgensen (35) finds a considerable increase in permeability of frog skin to both water and salt during moulting, together with increased salt uptake. A rapid increase in water uptake is noted in whole frogs upon exposure to low temperatures, followed by a slow increase which parallels an

increase in active salt uptake (36). Salt depleted frogs can take up salt from more dilute solutions than can normal animals (37). Linderholm (38) has studied ion permeability and electric conductivity of frog skin. Electrical permeability resistance (= DC resistance minus high frequency AC resistance) can vary widely with no change of net ionic outflux. A derived characteristic termed "reduced electrical resistance" does correlate over a limited range with ion flux.

Flexner, Cowie & Vosburgh (39) have summarized their studies relating to capillary permeability. Using Cl^{35} , Na^{24} , and DHO they conclude that the capillary wall of the guinea pig is 2.3 times as permeable to water as to sodium and chloride. Diffusion appears to be the predominant process concerned. Morel & Marois (40, 41) also note a rapid equilibration of Na^{24} between blood and tissue fluid.

A rapid exchange of Na^{24} and DHO across the placental barrier has been described by Flexner *et al.* (42) and Hellman *et al.* (43) in the human female and the guinea pig. Permeability increases markedly during gestation with water permeability always greater than that for salt. The rate of renewal of sodium in amniotic fluid is also very rapid (44). Placental interchange has been the subject of a review (45). Bárány (46) reports that the entrance of Na^{24} into the aqueous humor of the rabbit is independent of arterial pressure, thus indicating that ultrafiltration is not of paramount importance. The penetration of Na^{24} and chloride into cerebrospinal fluid has been studied (47).

A permeability of the gastric mucosa to hydrogen ions, as distinct from active secretion, has been demonstrated by Turner (48), who has shown that back-diffusion of the ions from the secretory side of gastric mucosa is probably a continuing process during both rest and activity. The rate of back-diffusion is a linear function of acid concentration gradient.

Flexner & Flexner (49) followed sodium and chloride changes in guinea pig cerebral cortex during foetal development and note that extracellular space (nonchloride) decreases sharply after about 40 days of gestation. At about that time, sodium in excess of chloride is found and the authors suggest that this indicates developing permeability to sodium. Since electrical activity is also noted at about this time, it seems possible to the reviewer that the excess sodium in the nerve cells may represent, not a change in permeability, but a new steady state level due to intermittent action of an outward sodium transport mechanism; whereas, prior to nervous activity, uninterrupted outward transport was the rule.

Lansing *et al.* (50) demonstrate a rapid exchange of Ca^{45} in the soft tissues of mice. This is regarded as an ion exchange phenomenon, and certain hyperplastic and carcinomatous conditions of the epidermis lead to decreased exchange.

Single cells.—A number of studies have been made of the permeability of the erythrocyte to inorganic elements commonly present. In this connection, special attention is called to the important series of papers by Ponder (51 to 56). Space does not permit detailed discussion. Ponder has been

concerned primarily with tonicity volume relationships of erythrocytes and offers extensive data on loss of potassium under a variety of conditions.

Greig & Holland (57, 58) have noted effects of acetylcholine and anticholinesterases on permeability of erythrocytes to potassium. For frog erythrocytes, Harris & Burn (17) report a permeability of 9×10^{-6} cm. per hr. for potassium or 2×10^{-6} cm. per hr. for sodium. Other papers dealing with erythrocytes will be mentioned later (Table I).

TABLE I
CELL, TISSUE, OR ORGANISM PERMEABILITY

Object	Substance or Phenomenon	Comments	Authors
Yeast	cobalt	600X accumulation effect of nickel	Nickerson & Zerahn (143)
Yeast	phosphate	600X accumulation effect of nickel	Batta & LeCoq (144)
Erythrocytes	anions	narcotic effects	Liebe (145)
Erythrocytes	hemolysis	organic calcium effects	Hahn & Bruns (146)
Erythrocytes	hemolysis	histamine effects	Zacco & DeVita (147)
Erythrocytes	hemolysis	Q ₁₀	Luckner (148)
Erythrocytes	hemolysis	glycolytic poisons	Rummel (149)
Sea urchin egg	osmotic behavior	ultraviolet effects	Reed (150)
Insect egg	iodine	development	Slifer (151)
Insect egg	water loss	development and temperature	Beament (152, 153)
Insect larvae	trivalent arsenic	development	Ricks & Hoskins (154)
Insect larvae	potassium chloride, urea, etc.	electrical and chemical studies	Richards & Fan (155)
Tetrahymena	succinate	increase with cyclopentane derivatives	Seaman & Houlihan (156)
Muscles	osmotic behavior	effects of adrenalectomy	Angerer & Angerer (157)
Bacteria	proflavin	concentration effects	Jackson & Hinshelwood (158)
Synovial membrane	phenol sulfonaphtalein	effects of hyaluronidase and steroids	Seifter <i>et al.</i> (159)
Various tissues	osmotic behavior	effects of hyaluronidase and steroids	Seifter <i>et al.</i> (160)
Capillary	protein	oxygen effects	Henry <i>et al.</i> (161)
Skin and Chara	salicylates	cation effects	Halpern <i>et al.</i> (162, 163)
Tooth enamel	methylene blue	morphological and electrical studies	Atkinson (164)

Wilson & Manery (59) have published an interesting study showing the permeability of rabbit leucocytes to sodium, potassium, and chloride. Permeability to all three elements could be demonstrated by following concentration changes within the cells, with a rapid, nearly complete exchange of Na²⁴. A noteworthy study by Abelson & Duryee (60), which will be mentioned in more detail later, shows the rapid entrance of Na²⁴ into frog ovarian eggs.

Cowie *et al.* (61), using Na²⁴ and K⁴², conclude that *Escherichia coli* is freely permeable to both elements. Studies on the exchange of potassium

ion and hydrogen ion by yeast have been continued by Conway (62, 63). Ørskov (64) concludes that potassium uptake by yeast is to be related to a primary energy source in the environment rather than to any specific nutrient or to the formation of any special product of assimilation. Schmidt *et al.* (65) record the interesting observation that, while yeast is assimilating phosphate from a normal medium, potassium is taken up; but, in the absence of magnesium ion, sodium is taken into the cells in excess.

A very extensive and valuable summary is given by Malm (66) of her studies on permeability of yeast to sugars, fluoride, and other substances.

ACCUMULATION AND ACTIVE TRANSPORT OF IONS

The reviews of Ussing (9), Krogh (67) and Rosenberg (68) should be referred to for basic discussions. Since all available evidence indicates a permeability of muscle, nerve, and some other cell types to the major inorganic elements of the environment, with at least a small part of the penetration in a form leading to real internal concentration changes (i.e., free diffusion, ion transport other than simple exchange phenomena) it is imperative to consider, first, the evidence for the need of active ion transport and second, possible mechanisms.

Conway [cf. (69)] has developed, with great care, a concept involving the exclusion of sodium and the passive distribution of potassium and chloride in muscle fibers, showing that high internal potassium is a necessary consequence of sodium exclusion. Ussing (5, 9) also gives calculations which indicate a passive distribution of potassium. Granting these considerations, it follows that sodium must be excluded by an impermeability of the membrane or by active outward transport or, most probably, by a combination of the two.

If a true impermeability is the basis for the ion distribution, then it follows that if a cell once admits, for example, sodium to which it is normally impermeable, there can never be any recovery of the original condition. On the other hand, if the apparent impermeability (see Teorell's comments on "false impermeability") is achieved by active outward transport of sodium, recovery would be possible and, indeed, necessary.

Unfortunately, most studies showing loss of potassium from cells and gain of sodium have not been extended to the reverse processes. The outward movement of potassium and inward movement of sodium upon, for example, stimulation, could be simply a passive diffusion due to chemical gradients in a system whose vital activity was momentarily decreased. The reverse process, however, demands a selective and active transport system, presumably for sodium (16).

Conway & Hingerty (70) report on recovery of rats whose muscles were made low-potassium and high-sodium by feeding a potassium deficient diet. Upon return to a normal diet, potassium levels were rapidly restored; sodium levels returned to normal only after several days. The authors conclude that there is a very slow outward transport of sodium with the muscle

fiber normally virtually impermeable to sodium ions. This result raises interesting questions, since earlier work by Fenn & Cobb (71) on similar animals showed a nearly comparable loss of potassium and gain of sodium during an hour or so period of stimulation, with a half time of recovery during rest of only 1 to 3 hr. for both sodium and potassium. It is possible, though not indicated by experimental data, that sodium and potassium shifts during dietary alterations are different from those during stimulation and recovery or perhaps the potassium deficient diet was deficient in other unknown factors concerned with sodium transport. The pathological changes noted in excessively high-sodium, low-potassium diets may be relevant (72).

The striking case of reversible exchange of sodium and potassium has been further examined by Flynn & Maizels (73) in human erythrocytes. Low temperature storage results in loss of potassium and gain of sodium. The changes are reversed upon incubating the cells at a higher temperature in the presence of glucose. The authors speculate briefly on the relationships of human erythrocytes to those of other species of mammals normally containing high sodium, pointing out that either an increase of permeability or a decrease of outward transport of sodium could account for the difference. While Flynn & Maizels (73) conclude that an outward transport of sodium ion is the major active process, Ponder (74), considering new as well as old evidence, suggests an active accumulation of potassium may also be operative.

Studies on acid secretion, while pertinent to the present discussion, are reported in other reviews dealing with the digestive system. Special reference should be made to the studies of electrical and metabolic factors by Davies & Ogston (75), secretion and pressure by Davies & Turner (76) and papers of more general scope on mechanisms of acid formation by Conway (62, 63).

BOUND AND NONEXCHANGEABLE INORGANIC ELEMENTS

Suggestions that ion accumulation may relate to specific binding by cell constituents are made frequently and isotope studies in some instances show incomplete exchange. Stone & Shapiro (77) ultrafiltered muscle brei and claimed to have demonstrated a potassium binding by the non-filterable components with complete diffusibility of sodium. Unfortunately, the published figures referred only to analyses of filtrates, hence it is impossible to assess the possibility that contamination, especially with sodium, was not operative. Carr & Topol (78), using membranes as reversible electrodes, determined sodium and chloride activity in solutions with and without protein present. At acid pH values, neither gelatin nor casein influenced inorganic ion activity. At alkaline pH values, however, measurements indicated up to 25 per cent sodium binding. It seems possible to the reviewer that an increase in ionization of phosphate groups of casein might account for the apparent binding.

The study by Abelson & Duryee (60) showed only a 12 per cent initial

exchange of sodium in frogs eggs, followed by a very slow continued uptake of isotope. Washing out experiments showed essentially similar results, initial movements of sodium in either direction being rapid. Radio-autographs failed to show any morphological compartmentalization to account for the two sodium fractions, although the nucleus exchanged proportionally more Na^{24} than the cytoplasm. Rothenberg (18) found about a 10 per cent exchange of potassium in squid nerve and a complete equilibrium of sodium, and Harris & Burn (17) report complete exchange of both sodium and potassium in frog muscle.

Thus, in different cell types, sodium and potassium are reported as either completely or incompletely exchangeable. To the reviewer's knowledge there is no good suggestion to account for these compartmentalizations, since, in view of the high electrical conductivity of protoplasm, simple ion binding (i.e., marked decrease in activity) would not appear to be plausible. Striated muscle fibers have a visible morphological compartmentalization (79) but they show complete exchange of potassium. Squid nerve axoplasm shows little morphological differentiation that could be invoked, especially since neurotubules appear not to exist as normal components of axoplasm (80). Roberts *et al.* (81) suggest that the high potassium content of *E. coli* relates to a binding by hexose phosphates, but the reviewer knows of no justification in the chemistry of these compounds to indicate such a binding. Since death of muscle or nerve fibers leads to a rapid equilibration with the inorganic elements of the environment, some very dynamic but exclusive system would seem indicated to account for compartmentalization of the materials in question.

It is regrettable that more precise information about ion binding, particularly of the alkali metals, is not available. While it seems unlikely that the large concentration differences noted for intracellular and extracellular potassium can be related to simple ion binding, from the physiological evidence available, specific binding of ions such as sodium as contrasted to potassium is strongly indicated. In one instance, at least, in inorganic chemistry, a compound which may be analogous to biologically important compounds binds sodium firmly but not potassium (82). It seems probable that selective binding (8) of ions at various interfaces, cell surfaces, and intracellular surfaces will be found to be increasingly important in future work. Hutner (83, 84) has discussed possible roles of "chelating" agents in this respect. Special attention should also be drawn to the solubilizing action of various phosphate compounds as studied recently by Neuberg & Mandl (85) and Neuberg & Roberts (86). Eddy co-workers (87) discuss potassium uptake by *Bacterium lactis aerogenes* in terms of specific binding at active centers necessary for growth.

PERMEABILITY TO ORGANIC SUBSTANCES

There have been relatively few studies, in the classical tradition, of comparing permeability of cells to members of a series of organic compounds.

Green (88) has made a precise spectroscopic study of the penetration of fatty acids into erythrocytes. Flexner *et al.* (89) studied the permeability of guinea pig capillaries to radioactive ferric β -globulin, finding its passage about 100 times slower than water. Uptakes of dyes (90) and enzymes (91) by yeast have been studied. The passage of various penicillin preparations through the placenta is reported (92) and also the kinetics of penetration of anti-helminthics into *Ascaris* (93). The abilities of various Nematode worms to take up P^{32} from host tissues are compared (94).

Jacobs *et al.* (95) have summarized their studies on hemolysis of erythrocytes of various vertebrates. Instructive diagrams illustrating relationships with respect to penetration of several organic solutes are presented. A critical discussion of the detection of osmotic abnormalities of erythrocytes is also given (96).

Electron microscope studies by Lindeman (97) of erythrocyte ghosts after osmotic hemolysis show that membranes remain intact during loss of hemoglobin. Jung (98) has reviewed recent work on the structure of the erythrocyte with special reference to the state of hemoglobin.

ACCUMULATION AND TRANSPORT OF ORGANIC SUBSTANCES

In an important paper, Stern *et al.* (4) report on the uptake and accumulation of glutamate by brain slices. An inward transport occurs against a gradient. Glucose is the best substrate for supporting the transport but some others will suffice. The paper should be consulted for details. Using kidney slices, Cross & Taggart (99) have studied uptake of *p*-aminohippurate in similar fashion. LeFevre (100) reports that copper and various sulphydryl agents depress permeability of erythrocytes to glycerol and sugar, the effects of some of the agents being reversed by glutathione and cysteine. He suggests surface phosphorylation as a first step in the passage of sugar, glycerol, and like substances across the membrane. Jacobs (101) discusses these and similar results in a recent review.

Accumulation and transport of organic materials, as related to kidney function and intestinal absorption are treated elsewhere (102, 103).

PERMEABILITY TO WATER AND OSMOTIC BEHAVIOR

A recent series of discussions includes consideration, among other things, of water movements in plants (104, 105) and through insect cuticle (106, 107). While the experimental observations are most interesting, theories regarding mechanisms involved in what is apparently a true active transport of water are neither complete nor simple of comprehension. The discussions cited should be read by all interested in the subject of water movement and permeability.

Water relations of plant cells are also considered by Thoday (108) and Arens comments briefly on active water transport, invoking electrical forces (109). Seeman (110) finds, using plasmolysis methods, a decrease in permeability to water of plant cells in low or high pH, with a plateau region of nor-

mal permeability around neutrality. The interesting movements of water in *Nitella* cells, discussed in the last review, have been further investigated by Osterhout (111, 112). Regional internal differences of osmotic pressure are related to water movements through the cells. The same author (113) reports on abnormal protoplasmic patterns in *Nitella* upon slight plasmolysis. Also, passage of electric currents through *Nereis* eggs causes marked water uptake, apparently due to swelling of cortical granules (114).

Opie (115) attempts to define various isotonic solutions by noting volume changes in tissue chunks. Using the term "isotonic" in its classical sense (= solution in which cells do not change volume) great diversity is shown for different tissues in different salt solutions. In this connection, the reviewer wishes to note that there is considerable difference in usage of the word "isotonic," most medical texts adopting the definition noted above and many research papers using it to designate solutions of freezing point depression equal to that of the normal environment of animal cells. Ponder (53) uses "isoplethechontic" to characterize solutions in which cell volume is maintained, a word which sacrifices euphoniousness to accuracy.

Shapiro (116) finds the nonsolvent space (*b* value) for *Arbacia* and *Chaetopterus* eggs to change upon fertilization, the decrease and increase respectively roughly paralleling changes in Q_{O_2} . Further studies attempting to define the physiological significance of this "constant" would be desirable.

In a review on the biology of viruses, Anderson (117) notes the apparent osmotic behavior of certain of the bacteriophages (T_2 , T_4 , T_6) which seem to possess membranes. Rapid shifts from high to low salt environments cause inactivation, slow changes do not. The inference can be made that the viruses are permeable to water and slowly permeable to salts. Wilbrandt (118) has investigated further "colloid osmotic hemolysis," a term he introduced to describe the osmotic hemolysis due to increased cation permeability of erythrocytes.

PHOSPHATE UPTAKE BY CELLS

This subject, while treated in other reviews [e.g. (119, 120, 126)], is mentioned briefly here because of the possible use of mechanisms of phosphate uptake as model systems for assimilation of other, especially inorganic, components of the environment. Previous reference has been made to the work of Spiegelman & Kamen (7) indicating that uptake of phosphorus by yeast involves combination with organic material at or near the surface of the cell. Kamen & Spiegelman (120) should also be consulted. Interesting papers by Rothstein and co-workers (121, 122) supply information which should be of great value in elucidating mechanisms of active uptake of material by living cells. Among other things, it seems to the reviewer that the surface localizations of esterases, aside from possible participation in synthesis, may act as safety controls to prevent undue amounts of metabolic intermediates from entering the cell from the outside. Yeast can utilize hexose phosphate of the environment when surface enzymes are intact, phosphate

appearing in the medium. Poisoning of the surface enzymes stops utilization of sugar phosphates, although sugar can still be metabolized (123).

Phosphate and glucose assimilation appear to be interdependent in yeast (124). Schmidt *et al.* (65) note that normal assimilation of phosphate requires potassium in the medium. The rate of uptake of P^{32} and rate of growth of *E. coli* are parallel, most of the intracellular P^{32} being nondialyzable (125).

Sacks (126) has reviewed his work indicating that the uptake of phosphate by muscle fibers is an active process involving formation of organic phosphate at the cell surface. An interesting report by Goffin *et al.* (127) gives the results of seeding a lake with P^{32} . Plankton took up the element rapidly, fish after about 50 hr., and marginal trees after about two weeks. Villee *et al.* (128) have reported on the uptake of P^{32} by sea urchin eggs and its intracellular distribution.

MODEL SYSTEMS AND THEORY

An informative and interesting discussion of the diffusion of ions across phase boundaries is given by Davies (129). The role of diffusion of solvate molecules away from solute when the latter enters a new phase is especially emphasized. Due to these complications, diffusion through the surface may be very slow as compared to diffusion in the bulk of any one phase. Activation energies for diffusion of potassium salts through cupric ferrocyanide membranes are reported by Tolliday *et al.* (32) to be in the neighborhood of 5,000 cal. per mol. as compared to around 4,000 for free diffusion in water. Space does not permit extensive review of the papers by Sollner & Gregor (130, 131) on "permselective" membranes and by Sollner (132, 133) on "bi-ionic potentials." Weatherby (134) has studied an artificial phospholipid membrane. Lipid and lipoprotein contributions to membrane properties of cells have been discussed by Chargaff (135), Ponder (136), and Booij (137).

Barrer & Jost (138) comment on interstitial diffusion, noting that diffusion coefficients in zeolite-like systems may decrease as the interstitial concentration of sorbate increases. The role of diffusion in the combination of chlorine with wool has been treated by Alexander *et al.* (139). Hartley & Crank (140) discuss fundamental concepts and definitions of diffusion. Hearon (141) treats mathematically cases of simultaneous diffusion streams, showing the degrees of interactions to be expected [cf. (2)].

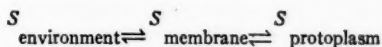
Using hemoglobin as an indicator, Müller (142) has studied the diffusion of oxygen through water and hemoglobin solution layers. The movement of gas through water layers follows Fick's law well, although the diffusion constant varied with hemoglobin concentration. Diffusion rates through hemoglobin layers were lower than through water, for stationary gradients the ratio of rate through water to rate through 16 per cent hemoglobin being 1.5.

COMMENTS

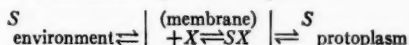
It is the opinion of the reviewer that concepts of permeability have been clouded by oversimplification. Given a substance *S*, placed in the environ-

ment of the cell, then that substance could enter the aqueous interior protoplasm by several mechanisms.

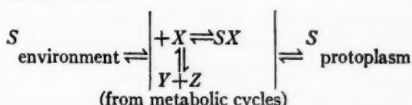
1. The substance could penetrate through holes or by solution in the membrane.



2. The substance could form a specific complex.



3. The complex could be energetically formed and/or broken down (i.e., coupled to oxidative systems).



In case 1, the conditions are the simplest and movement of S should proceed to an equilibrium and at a rate defined by the usual rules of diffusion. In case 2, a possible high degree of specificity is added plus the restriction that complex formation might limit the rate of movement of S , rather than concentration (=electrochemical) gradients. In case 3, there is a possibility of active selective transport. The conditions of case 1 are assumed to hold by many workers, usually not explicitly. Formation of un-ionized complexes (case 2) has long been advocated by Osterhout to account for selective ion uptake by *Nitella* and *Valonia*. The conditions for case 3 have perhaps been most explicitly stated for the instance of resorption of sugar by the kidney tubules.

A cell could presumably be completely impermeable to a substance as defined in case 1, while showing rapid exchange and/or transport according to cases 2 and 3. From the literature cited, it is obvious that there is a distinct tendency for recent observations to be interpreted in terms of 2 and 3 rather than 1. In other words, much evidence is consistent with the view that many substances penetrate by first being incorporated into the substance of the cell (assimilation). Whatever the merits of this point of view, it is more satisfying to the physiologist, since a substance entering the cell through a hole seems quite uninspiring as compared to a substance combined with other protoplasmic constituents.

It is, of course, necessary to point out that terms such as "uptake," "complex formation" and "active transport" can be very dangerous if banded around loosely. A primary requirement, before invoking the terms, should be that of showing that the "simple diffusion" of case 1 does not work.

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